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# CONTENTS OF VOLUME 158

No. 1, MARCH, 1945

PAGE

VALLERSTEIN, JAMES S., and STERN, KURT G. On bisulfite as an inhibitor of carboxylase and the mechanism of glycerol fermentation	1
LOFF, RUTH L., and STERN, KURT G. On extracts of carp spleen and their effect on carboxylase and cocarboxylase	19
SCHOFF, FRITZ. The temperature coefficient for the denaturation of chorionic gonadotropin by urea	29
ARMANN, HEINZ, and MOSES, SYLVIA G. Content and state of glutathione in the tissues of the eye	33
ARMANN, HEINZ, and MOSES, SYLVIA G. The cytochrome oxidase activity of the lens of the bovine eyes	47
ENTL, ALBERT A., and PAGE, IRVINE H. The purification of angiotonin	49
ITCH, FLETCHER P., JR., and MILONE, HENRY S. Studies on the sex hormones. I. The quantitative estimation of estrone in pure solution	61
ARBY, WILLIAM J., DEMEO, R. H., BERNHEIM, MARY L. C., and BERNHEIM, FREDERICK. The metabolism of phenolic compounds by normal and scorbutic guinea pig liver slices <i>in vitro</i>	67
EMIN, DAVID, and RITTENBERG, D. On the mechanism of the conversion of ornithine to proline <i>in vivo</i> ; intramolecular nitrogen shift	71
ZIEWIATKOWSKI, DOMINIC D., and LEWIS, HOWARD B. The metabolism of trimethylacetic (pivalic) and tertiary butylacetic acid. New examples of conjugation with glucuronic acid	77
ODGSON, ROLAND, RIKER, A. J., and PETERSON, W. H. Polysaccharide production by virulent and attenuated crown-gall bacteria	89
LANESE, ANTHONY A. The utilization of <i>D</i> -amino acids by man. II. Cystine	101
IACFADYEN, DOUGLAS A. Estimation of formaldehyde in biological mixtures	107
AMECNIK, PAUL C., STEPHENSON, MARY L., and COPE, OLIVER. Peptidase activity of lymph and serum after burns	135
CRUEGER, KEATHA, and PETERSON, W. H. The response of <i>Lactobacillus casei</i> and <i>Streptococcus faecalis</i> to vitamin B <sub>12</sub> and thymine	145
SACHMANSOHN, DAVID, and JOHN, HEDDA M. Studies on choline acetylase. I. Effect of amino acids on the dialyzed enzyme. Inhibition by $\alpha$ -keto acids	157
GREHL, W. A., and ELVEHJEM, C. A. The importance of "folic acid" in rations low in nicotinic acid	173
JALDWELL, M. J., and PARRISH, D. B. The effect of light on the stability of the Carr-Price color in the determination of vitamin A	181
MARSHALL, WILFRID, and MARSHALL, CHARLES R. The action of nitrites on blood	187
LIFSON, NATHAN, and LORBER, VICTOR. The time required for establishment of the thermal steady state in the Hill-Baldes apparatus, with application to the more rapid determination of osmotic activity	209
LIFSON, NATHAN, LORBER, VICTOR, and HILL, EDWARD L. Determination of deuterium oxide by means of the Hill-Baldes vapor tension apparatus	219
FRIEDLANDER, H. D., CHAIKOFF, I. L., and ENTENMAN, C. The effect of ingested choline on the turnover of plasma phospholipids	231
SIMS, E. A. H. Microdetermination of glycocyamine and arginine by means of a synthetic ion exchange resin for chromatographic separation	239

MILITZER, WALTER E. The inhibition of carbohydrate oxidations by borate . . .	247
COHEN, SEYMOUR S. The enzymatic degradation of thymus nucleohistone. . .	255
HOCHBERG, MELVIN, MELNICK, DANIEL, and OSER, BERNARD L. Chemical determination and urinary excretion of the metabolite N <sup>1</sup> -methylnicotinamide . . .	265
CHRISTENSEN, HALVOR N., UZMAN, LAHUT, and HEGSTED, D. MARK. A note on the amino acid composition of tyrocidine . . .	270
LICHE LBRENGER, LILLIAN, and BROWN, JEAN D. The fat, water, chloride, total nitrogen, and collagen nitrogen content in the tendons of the dog . .	283
SCOTT, M. L., NORRIS, L. C., HEUSER, G. F., and BRUCE, W. F. Studies on organic factors required for prevention of anemia in chicks . . .	291
PILLYMER, LOUIS, and HUTCHINSON, M. C. The determination of the albumin and globulin contents of human serum by methanol precipitation . . .	299
BRIGGS, G. M., JR., LUCKEY, T. D., ELVENJEM, C. A., and HART, E. B. Studies on vitamins B <sub>12</sub> and B <sub>11</sub> and related substances in chick nutrition . . .	303

### *Letters to the Editors*

KALCKAR, HERMAN M. Enzymatic microanalysis of purine compounds . . .	313
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### No. 2, APRIL, 1945

GREEN, ARDA ALDEN. The diffusion constant and electrophoretic mobility of phosphorylases <i>a</i> and <i>b</i> . . .	315
CORI, GERTY T., and CORI, CARL F. The enzymatic conversion of phosphorylase <i>a</i> to <i>b</i> . . .	321
CORI, GERTY T. The effect of stimulation and recovery on the phosphorylase <i>a</i> content of muscle . . .	333
CORI, CARL F., and CORI, GERTY T. The activity and crystallization of phosphorylase <i>b</i> . . .	341
ARCHIBALD, REGINALD M. Methods for the determination of alloxan, together with observations of certain properties of alloxan . . .	347
HAMILTON, PAUL B. Gasometric determination of glutamine amino acid carboxyl nitrogen in plasma and tissue filtrates by the ninhydrin-carbon dioxide method . . .	375
HAMILTON, PAUL B. Glutamine a major constituent of free $\alpha$ -amino acids in animal tissues and blood plasma . . .	397
WEISHAUF, SIDNEY, MEDES, GRACE, and FLOYD, NORMAN F. Fatty acid metabolism. III Reactions of carboxyl-labeled acetic acid in liver and kidney . .	411
VAN WAGENFONSE, WILLEM J., and LAMERON, HILDEGARD. A dietary factor essential for guinea pigs VI. Changes in the distribution of acid-soluble phosphorus in the muscle during a deficiency of the antistiffness factor . .	421
BERNARD, EUGENE, and BERNEBER, MARY I. C. The inactivation of tyramine by heart muscle <i>in vitro</i> . . .	425
NORRIS, CARL R., and BENOIT, GEORGE J., JR. Studies on trimethylamine oxide. I. Occurrence of trimethylamine oxide in marine organisms . . .	433
BENOIT, GEORGE J., JR., and NORRIS, CARL R. Studies on trimethylamine oxide. II. The origin of trimethylamine oxide in young salmon . . .	439
NORRIS, CARL R., and BENOIT, GEORGE J., JR. Studies on trimethylamine oxide. III. Trimethylamine oxide excretion by the rat . . .	443
NORRIS, CARL R., and SIMMONS, RICHARD W. The hematopoietic activity of xanthopterin in young salmon . . .	449

BROWN, ELMER B., BINA, ALBERT F., and THOMAS, JAMES M. The use of diazotized <i>p</i> -aminoacetophenone in the determination of vitamin B <sub>6</sub> (pyridoxine)	455
QUIGLEY, JAMES J., and MURASCHI, THELMA F. Amino acid inhibition of copper proteinate formation	463
KREKE, CORNELIUS W., BARTLETT, SISTER MARY DOMITILLA, and SMALT, SISTER MARIE AUGUSTINE. An accelerator of catalase activity	469
SOBEL, ALBERT E., ROCKENWACHER, MORRIS, and KRAMER, BENJAMIN. Carbonate content of bone in relation to the composition of blood and diet	475
CUNNINGHAM, ELIZABETH, and SNELL, ESMOND E. The vitamin B <sub>6</sub> group. VI. The comparative stability of pyridoxine, pyridoxamine, and pyridoxal	491
SNELL, ESMOND E. The vitamin B <sub>6</sub> group. VII. Replacement of vitamin B <sub>6</sub> for some microorganisms by d(-)-alanine and an unidentified factor from casein	497
LIPMANN, FRITZ, and TUTTLE, L. CONSTANCE. On the condensation of acetyl phosphate with formate or carbon dioxide in bacterial extracts	505
UTTER, M. F., LIPMANN, FRITZ, and WERKMAN, C. H. Reversibility of the phosphoroclastic split of pyruvate	521

### *Letters to the Editors*

MILONE, HENRY S., PESARE, PASQUALE J., and VEITCH, FLETCHER P., JR. Enhancement of antibody production	533
WHITE, WILFRED, and KOCH, F. C. Interference by cystine with the quantitative nitroprusside test for methionine	535

### NO. 3, MAY, 1945

ZAMECNIK, PAUL C., LAVIN, GEORGE I., and BERGMANN, MAX. Microphotometric determination of the rate of enzymatic proteolysis	537
BANERJEE, SACHCHIDANANDA. On the hypoglycemic action of alloxan	547
SNELL, ESMOND E., and SHIVE, WILLIAM. Growth inhibition by analogues of pantothenic acid. Pantothenyl alcohol and related compounds	551
LUECKE, R. W., and PEARSON, P. B. Effect of the ingestion of excessive quantities of choline on the amount in tissues and urine	561
DAVENPORT, HORACE W. The inhibition of carbonic anhydrase by thiophene-2-sulfonamide and sulfanilamide	567
RUEGGER, W. R., MICHAUD, L., and ELVEHJEV, C. A. A simplified method for the determination of iron in milk	573
BISCHOFF, FRITZ. Note concerning the reversible inactivation of prolan	577
BONSNES, ROY W., and TALSKY, HERTHA H. On the colorimetric determination of creatinine by the Jaffe reaction	581
CHRISTENSEN, HALVOR N., and HEGSTED, D. MARK. The course of the acid hydrolysis of gramicidin	593
BROWN, HERMAN. The determination of uric acid in human blood	601
RICE, RANDALL G., BALLOU, GERALD A., BOYER, PAUL D., LUCK, JAMES MURRAY, and LUM, FUNSTON G. The papain digestion of native, denatured, and "stabilized" human serum albumin	609
JOHNSON, B. CONNOR, HAMILTON, T. S., and MITCHELL, H. H. The excretion of pyridoxine, "pseudopyridoxine," and 4-pyridoxic acid in the urine and sweat of normal individuals	619
MITCHELL, H. H., HAMILTON, T. S., STEGGERDA, F. R., and BEAN, H. W. The chemical composition of the adult human body and its bearing on the biochemistry of growth	625

RUBIN, SAUL H., and DE RITTER, ELMER. The adsorption of riboflavin on florasil .. . . .	639
PFACE, JAMES W., MILLER, RUSSELL C., DUTCHER, R. ADAMS, and THORP, WILLIAM T. S. The thiamine content of pig blood .. . . .	647
NACHMANSOHN, DAVID, and ROTHENBERG, MORTIMER A. Studies on cholinesterase. I. On the specificity of the enzyme in nerve tissue .. . . .	653
RATHBUN, EDITH N., and PACE, NELLO. Studies on body composition. I. The determination of total body fat by means of the body specific gravity... ..	667
MORALLS, MANUEL F., RATHBUN, EDITH N., SMITH, ROBERT E., and PACE, NELLO. Studies on body composition. II. Theoretical considerations regarding the major body tissue components, with suggestions for application to man .. . . .	677
PACE, NELLO, and RATHBUN, EDITH N. Studies on body composition. III. The body water and chemically combined nitrogen content in relation to fat content .. . . .	685
KREJCI, LAURA E., SWEENEY, LUCILE, and SANIGAR, EDWARD B. The electrophoretic analysis of stored liquid human plasmas .. . . .	693
BRODIE, BERNARD B., and UDENFRIEND, SIDNEY. The estimation of basic organic compounds and a technique for the appraisal of specificity. Application to the cinchona alkaloids .. . . .	705

# *Letters to the Editors*

LYLE, PAUL D. The prevention by caprylate of urea and guanidine denaturation of serum albumin .. . . .	715
UBERGLER, A. The lysine content of casein and zein .. . . .	717
MASON, HAROLD L. Isolation of a urinary steroid with an oxygen atom at carbon 11 .. . . .	719
DEARKEIN, DAVID L. Unaltered globin, and crystalline, synthetic (reconstituted) myoglobin .. . . .	721
KALICKAR, HERMAN M. Enzymatic synthesis of a nucleoside .. . . .	723
LEVIN, LOUIS. The isolation of $\alpha$ -estradiol from the urine of stallions .. . . .	725
COOPER, GERALD R. Electrophoretic analysis of mixtures of protein .. . . .	727
INDEX TO VOLUME 155 .. . . .	729

# ON BISULFITE AS AN INHIBITOR OF CARBOXYLASE AND THE MECHANISM OF GLYCEROL FERMENTATION\*

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(From the Overly Biochemical Research Foundation, New York)

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It will be shown in the present paper that sodium bisulfite, under certain well defined conditions, markedly inhibits the reaction, pyruvic acid  $\rightarrow$  acetaldehyde +  $\text{CO}_2$ , as catalyzed by yeast *carboxylase*, a diphosphothiamine magnesium-protein (1, 2).

For some time it has been known that bisulfite reacts chemically with several components of this enzyme-substrate system. Between pH 5 and 7, it will form stable complexes with pyruvic acid (3) and acetaldehyde (4), and within a similar pH range it will split cocarboxylase (5), just as free thiamine (6), into an insoluble pyrimidinesulfonic acid and the corresponding thiazole component. An inhibition of the enzymatic catalysis by this reagent could, therefore, be due either to the *irreversible* destruction of the *coenzyme* or to *reversible* complex formation with the *substrate*. Under the conditions of our experiments, the *bisulfite competes with the carboxylase for pyruvic acid*.

## EXPERIMENTAL

### *Materials and Methods*

The starting material was a batch of air-dried Krueger's bottom yeast. For experiments on *dry yeast*, weighed amounts of this material were evenly suspended in phosphate buffer, usually of pH 6.2, to yield a concentration of 20 mg. of dry yeast per cc. For experiments on *Lebedev juice* 1 part of dry yeast was evenly suspended in 3 parts of warm tap water. After the mixture had stood for 2 hours in the incubator at 37°, the thick suspension was centrifuged for 30 minutes at approximately 3200 R.P.M. in a horizontal centrifuge and the supernatant, translucent fluid was carefully decanted. 5 gm. of the dry yeast employed would yield about 7 cc. of highly active maceration extract when treated in this way. In the preparation of *purified carboxylase* another batch of Krueger's dry yeast was used, and the

\* These experiments form part of a research project on glycerol fermentation, conducted during 1942-44 under the auspices of the Soap and Glycerine Division of the Food Distribution Administration, United States Department of Agriculture. A preliminary account of this work was given before the Division of Biochemistry, American Chemical Society, Cleveland, 1944.

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procedure of Green *et al.* (1) was followed up to their Step 4. For the experiments on *apocarboxylase* (coenzyme-free carboxylase), the dry yeast was treated with alkaline phosphate and finally suspended in phosphate buffer, pH 6.2, as described by Lohmann and Schuster (5). The *cocarboxylase* used here was a synthetic preparation of Merck.

The *sodium bisulfite* employed in this work was Merck's reagent grade (guaranteed minimum purity 95 per cent). For experiments in which the exact concentration of  $\text{NaHSO}_3$  was important, the solutions were analyzed by iodometric titration; otherwise 1 M stock solutions were prepared from accurately weighed amounts of the salt, and dilutions were then made from this stock solution. In the majority of the experiments freshly prepared solutions were used. The pH of the 1 M stock solution was 5.2 as measured with the glass electrode.

The *pyruvic acid* was added to the test system in the form of its crystalline sodium or lithium salts which were analyzed for their actual content in pyruvate by iodometric titration in presence of bisulfite.

For the determination of *carboxylase activity*, the standard manometric method (5) was employed, with simple Warburg-Barcroft manometers and conical vessels of about 20 cc. total capacity. In several instances the results obtained by manometry were checked with the aid of colorimetric pyruvate determinations (7) in the reaction mixtures at the beginning and at the end of the manometric experiments.<sup>1</sup> The agreement between the two methods was satisfactory.

### *Arrangement of Experiments and Results*

The first experiments were designed to determine the effect of sodium bisulfite over a wide range, viz. at  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  M final concentration, on the carboxylase activity of dry yeast at pH 6.2 and at low pyruvate concentration ( $7.6 \times 10^{-3}$  M). In a typical experiment, 1 cc. of a Krueger dry yeast suspension in 0.1 M phosphate buffer, containing 20 mg. of dry yeast, and 1.7 cc. of 0.1 M phosphate, pH 6.2, were placed in the main compartment of a series of Warburg vessels equipped with two side arms. One of the side arms received 0.3 cc. of a sodium pyruvate-magnesium chloride solution, containing an equivalent of 2.2 mg. of pyruvic acid and 0.1 mg. of magnesium. The second side arm was filled with 0.3 cc. of  $\text{NaHSO}_3$  solution of varying strength (M, 0.1 M, and 0.01 M respectively). The control vessels contained no bisulfite. The gas space of the vessel was left filled with air. The manometers were placed in the thermostat and equilibrated for 7 minutes at  $25^\circ$ . The stop-cocks were then closed and shaking was continued for 20 minutes, the manometers being read every 10 minutes.

<sup>1</sup> The authors are indebted to Mrs. Gertrude D. Maengwyn-Davies and Miss Lucy Bergmann for the colorimetric and volumetric pyruvate analyses.

There was little or no change in gas pressure during this control period. Now the substrate was added to the yeast suspension from the first side arm. Immediately a positive gas pressure occurred in all manometers owing to the onset of the decarboxylation reaction and the liberation of  $\text{CO}_2$ . After 5 to 10 minutes the bisulfite was added to the reaction system from the second side arm and the manometer readings were recorded in 5 minute intervals for 30 to 50 minutes, depending on the rate of the reaction. It was found that  $10^{-3}$  M bisulfite, under these conditions, had little or no effect on the course of the reaction as compared with the control vessels, while  $10^{-2}$  M bisulfite produced a marked inhibition after a brief lag period of about 5 to 10 minutes, increasing in extent with time. The vessel containing the highest  $\text{NaHSO}_3$  concentration ( $10^{-1}$  M) showed a negative gas pressure, presumably caused by the autoxidation of the bisulfite which is known to be a chain reaction catalyzed by traces of copper. Similar results were obtained when Lebedev juice was substituted for dry yeast as a source of carboxylase. The progressively marked inhibition of the enzymatic reaction by  $10^{-2}$  M bisulfite proved to be a reproducible phenomenon throughout these experiments. In order to avoid the complications arising from the reaction of the bisulfite with molecular oxygen, the experiments were repeated in an atmosphere of pure nitrogen in which the activity of the enzyme itself remains unchanged. To assure completely anaerobic conditions sticks of yellow phosphorus were placed in the central well of the manometer vessels where they were half submerged in 0.3 cc. of water. Gassing the vessels with nitrogen (purified nitrogen of the Ohio Chemical Company) prior to the experiment was continued until no phosphorus luminescence was detectable in the dark, corresponding to oxygen pressures of less than  $10^{-5}$  atmosphere. Under these conditions,  $10^{-1}$  and  $10^{-2}$  M bisulfite produced an inhibition of the reaction leading to a virtual cessation about 12 minutes after adding the inhibitor, while  $10^{-3}$  M  $\text{NaHSO}_3$  again had no effect. The result of such an experiment is shown in graphical form in Fig. 1.

The inhibition of yeast carboxylase by  $10^{-1}$  and  $10^{-2}$  M bisulfite and the ineffectiveness of  $10^{-3}$  M  $\text{NaHSO}_3$ , with pyruvate in  $7.6 \times 10^{-3}$  M concentration, could be confirmed with purified carboxylase, prepared according to Green *et al.* (1), as well as with apocarboxylase (alkaline-washed dry yeast) plus synthetic carboxylase (Merck) as the catalysts. The same was true when the sodium pyruvate was replaced by an equivalent amount of lithium pyruvate as substrate, and also when the phosphate buffer of pH 6.2 was replaced by 0.3 M acetate buffer of pH 4.9. The reliability of the manometric method for the measurement of the bisulfite inhibition of the carboxylase-pyruvate reaction was controlled in several instances by performing colorimetric pyruvate assays by the method of Friedemann and



Haugen (7) on the reaction mixtures at the beginning and end of the manometric experiment. Thus, in one experiment with dry yeast in phosphate buffer of pH 6.2, with  $7.6 \times 10^{-3}$  M lithium pyruvate and  $10^{-2}$  M bisulfite, the inhibition, as determined *manometrically* in duplicate vessels, amounted to 46 per cent, while the *colorimetric* technique indicated an inhibition of

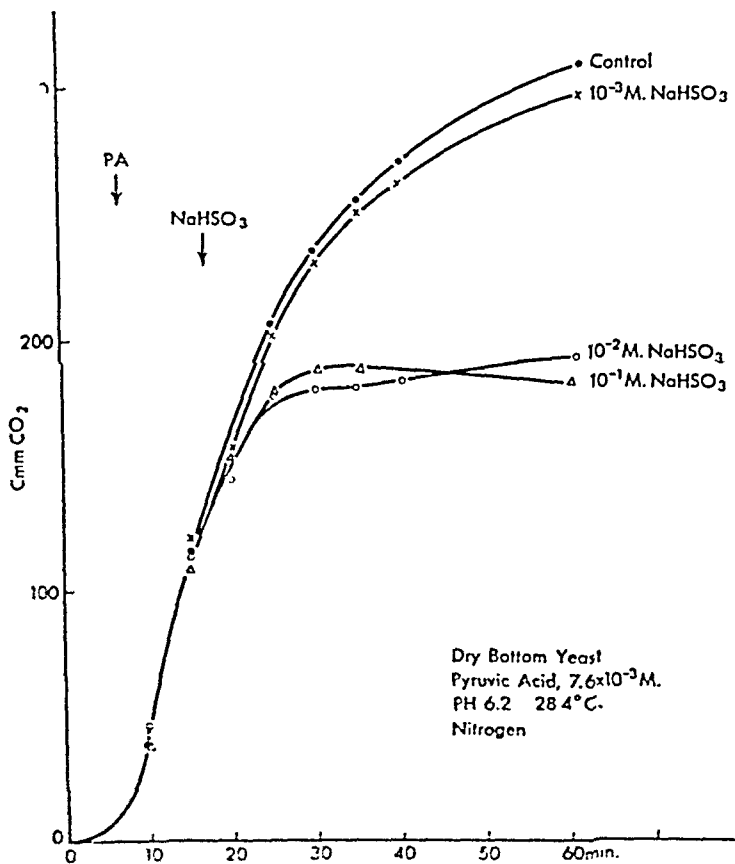


FIG. 1. Effect of bi-sulfite on carboxylase activity of dry yeast

40 and 32.5 per cent, i.e. a somewhat lower value of the same order of magnitude. In the same experimental series,  $10^{-1}$  M NaHSO<sub>3</sub> produced an inhibition of 41 and 59 per cent, as determined colorimetrically, no manometric values being available in this instance because of oxygen absorption by the bisulfite.

It will be noted that in the experiments outlined above no inhibition occurred when the bisulfite was present in low concentration ( $10^{-3}$  M) as compared with the pyruvate ( $7.6 \times 10^{-3}$  M), whereas a marked inhibition was observed at  $10^{-1}$  and  $10^{-2}$  M  $\text{NaHSO}_3$  when the inhibitor was present in stoichiometric excess over the substrate. In order to determine the rate of the reaction under conditions of equimolar concentrations of substrate and inhibitor, an experiment was performed with  $7.6 \times 10^{-3}$  M pyruvate and  $7.6 \times 10^{-3}$  M bisulfite. Under these conditions, an inhibition of 70 per cent was observed manometrically.

The results obtained in the experiments performed with  $7.6 \times 10^{-3}$  M pyruvate and varying bisulfite concentrations are summarized in Table I.

The data given in Table I fall short of an adequate description of the bisulfite effect because of the change in slope, with time, of the reaction curves obtained with the enzyme-substrate-inhibitor systems. They merely represent the differences in substrate turnover at the end of the observation period.

In the next series of experiments the substrate concentration was varied from  $3 \times 10^{-3}$  to  $7 \times 10^{-2}$  M pyruvate, while the inhibitor concentration was maintained constant at  $10^{-2}$  M bisulfite. It was found that at low substrate concentrations, at which the bisulfite was in considerable stoichiometric excess, the inhibition was most pronounced, although the absolute reaction rate of the control was relatively small owing to insufficient saturation of the enzyme with substrate. As the pyruvate concentration was increased, the absolute reaction rate also increased, while the degree of relative bisulfite inhibition diminished until, at the highest pyruvate concentration employed ( $7 \times 10^{-2}$  M), the bisulfite-containing system exhibited a somewhat higher reaction rate than the control (see Table II). In the experiment which is graphically reproduced in Fig. 2, the bisulfite caused an inhibition of 91 per cent in the system containing  $3 \times 10^{-3}$  M pyruvate, of 78 per cent with  $7.6 \times 10^{-3}$  M pyruvate, and of only 4 per cent with  $1.52 \times 10^{-2}$  M pyruvate. Fig. 2 also illustrates the dependence of absolute reaction rate on the substrate concentration.

In another experiment with  $1.55 \times 10^{-2}$  M lithium pyruvate and  $0.9 \times 10^{-2}$  M sodium bisulfite, the *manometric* readings indicated an inhibition of 7 per cent, whereas the *colorimetric* pyruvate determination yielded a value of 13 per cent for the inhibition.

Inasmuch as these experiments demonstrated that the extent of the carboxylase inhibition is a function of the bisulfite concentration at constant pyruvate concentration and a function of the pyruvate concentration at constant bisulfite concentration, it appeared desirable to compare the reaction rate in systems containing equimolar amounts of pyruvate and bisulfite and in systems containing identical amounts of pyruvate but no bisulfite at different absolute concentration levels. It was found that at

$1.2 \times 10^{-2}$  M pyruvate and pyruvate-bisulfite concentration respectively the inhibition caused by bisulfite amounted to 76 per cent, while at the

TABLE I

*Inhibition of Carboxylase by Bisulfite at  $7.6 \times 10^{-2}$  M Pyruvate Concentration*

Experiment No.	Enzyme preparation	pH	Atmosphere	NaHSO <sub>3</sub> concentration	Results of manometric determinations		
					With NaHSO <sub>3</sub>	Without NaHSO <sub>3</sub>	Inhibition
					microliters CO <sub>2</sub>	microliters CO <sub>2</sub>	per cent
1	Dry bottom yeast	6.3	Air	$10^{-2}$	299	317	6
2	" " "	~6.3	N <sub>2</sub>	$10^{-2}$	206		35
				$10^{-2}$	296	307	4
				$10^{-2}$	194		37
				$10^{-1}$	183		40
3	" " "	~6.3	Air	$7.6 \times 10^{-2}$	96	317	70
4	" " "	4.9	N <sub>2</sub>	$10^{-2}$	267	256	0
				$10^{-2}$	102		60
				$10^{-1}$	98		62
5	" " "	6.2	Air	$10^{-2}$	185	344	40
6	" " " + carboxylase	~6.2	N <sub>2</sub>	$10^{-1}$			(36)*
				$10^{-2}$	163	398	(50)*
				$10^{-1}$	13		59
7	Dry bottom yeast	~6.2	Air	$10^{-2}$	97†	327	97
					95		71
					102		69
					75		77
8	Lebedev juice, 0.1 cc. per vessel	6.3	"	$10^{-2}$	116	119	3
				$10^{-2}$	36		70
				$10^{-2}$	271	260	0
				$10^{-2}$	124		52
9	Purified carboxylase	~6.3	"	$10^{-2}$	317	318	0
				$10^{-2}$	175		45
10	Apocarboxylase and cocarboxylase	~6.3	"	$10^{-2}$	92	100	8
				$10^{-2}$	13		87
11	" " ‡	~6.3	N <sub>2</sub>	$10^{-2}$	163	161	0
				$10^{-2}$	60		63

\* By colorimetric pyruvate assay.

† Dry yeast in contact with the NaHSO<sub>3</sub> for 7, 17, 27, and 37 minutes respectively.

‡ Cocarboxylase in contact with NaHSO<sub>3</sub> for 74 minutes. During this period, the bisulfite concentration was 10 times that of the final value given above.

$1.2 \times 10^{-1}$  M level it was only 30 per cent (see Fig. 3). This experiment shows that the extent of bisulfite inhibition of carboxylase action is not

only a function of the *relative* but also of the *absolute* substrate and inhibitor concentrations (see Table II).

The next point of interest concerns the *reversibility* of the bisulfite inhibition. We therefore investigated the conditions under which this inhibition may be relieved once it has been established in the carboxylase-pyruvate system. The observation that the extent of the bisulfite inhibi-

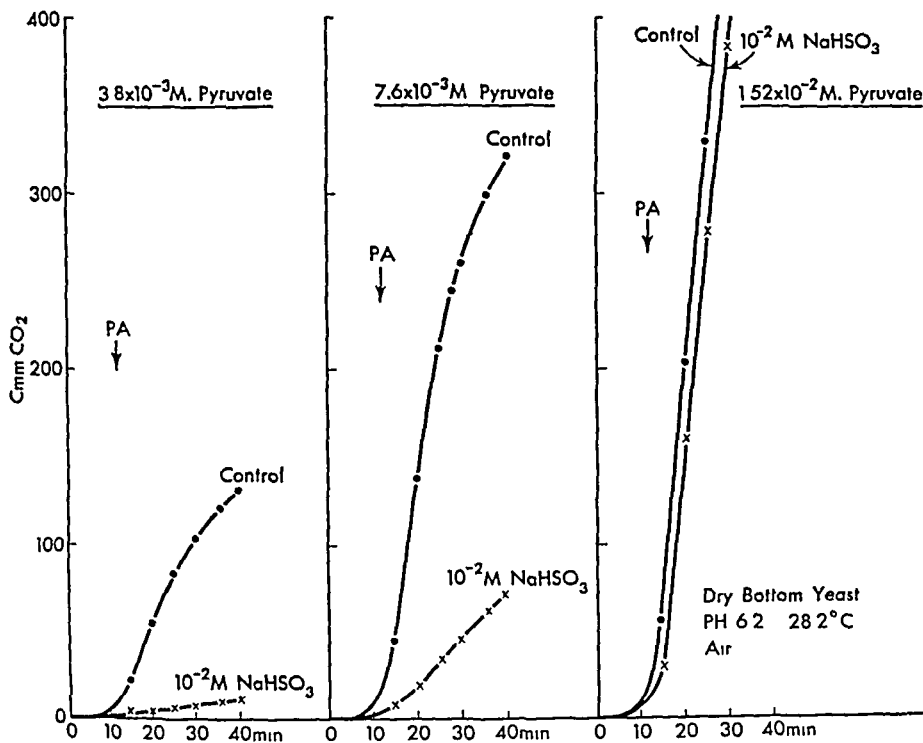


FIG. 2. Effect of varying the pyruvate concentration on the bisulfite inhibition of carboxylase action.

tion decreases with increasing substrate concentration suggested the use of pyruvate for this purpose. To this end, mixtures of phosphate buffer, pH 6.2, small amounts of pyruvate (2.2 mg. of pyruvic acid equivalent as Na salt), and varying amounts of bisulfite were placed in the main compartment of a series of Warburg vessels. One side arm of the vessels received a suspension of 20 mg. of dry yeast and the other an amount of pyruvate solution corresponding to 20 mg of free pyruvic acid. The central well

contained yellow phosphorus and water, the gas space pure nitrogen. When the enzyme preparation was added to the pyruvate-bisulfite mixtures in the main compartment, only the bisulfite-free control showed a rapid  $\text{CO}_2$  evolution, while the systems containing  $10^{-1}$  and  $10^{-2}$  M bisulfite were strongly inhibited (see Fig. 4). On addition of the large amount of pyruvate from the second side arm, the inhibition in the vessel containing  $10^{-2}$  M  $\text{NaHSO}_3$  was abolished, while that produced by  $10^{-1}$  M  $\text{NaHSO}_3$  was not ap-

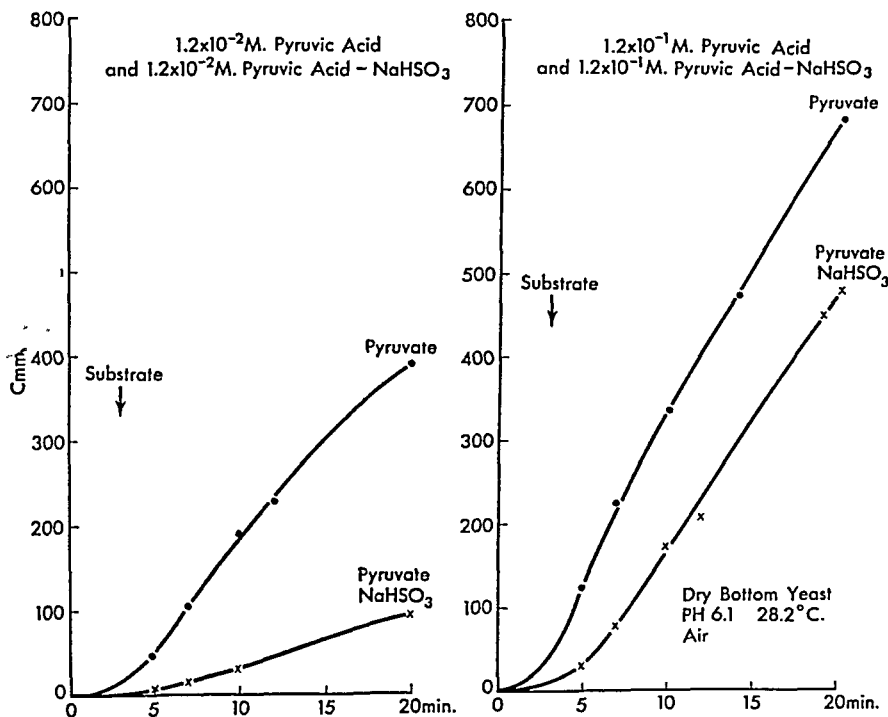


FIG. 3. Action of carboxylase on free pyruvate and on the pyruvate-bisulfite complex.

preciably affected, as shown in Fig. 4. It should be pointed out that the ultimate pyruvate concentration amounted to  $7.6 \times 10^{-2}$  M, thus leaving the bisulfite still in slight stoichiometric excess in the latter system. This experiment demonstrates the fundamentally reversible nature of the bisulfite inhibition.

It has been pointed out that bisulfite is known to react, under certain conditions, with several components of the carboxylase-pyruvate system.

The subsequent experiments were designed to elucidate further the mechanism of the bisulfite inhibition of the catalysis under the particular conditions obtaining in the present investigation. Thus, if the bisulfite exerts its inhibitory effect by a cleavage of the *coenzyme* (5), the addition of large amounts of cocarboxylase would be expected to relieve, at least temporarily, the bisulfite inhibition. As the experiment reproduced in Fig. 5 shows, the addition of 50  $\gamma$  of synthetic cocarboxylase failed to affect the carboxylase inhibition by  $10^{-1}$  and  $10^{-2}$  M  $\text{NaHSO}_3$ .

TABLE II

*Inhibition of Carboxylase by Bisulfite at Varying Pyruvate Concentrations*  
Conditions of manometric assay, dry bottom yeast, pH 6.2, 28°, in air.

Experiment No.	$\text{NaHSO}_3$ concentration	Pyruvate concentration	Results of manometric determinations		
			With $\text{NaHSO}_3$	Without $\text{NaHSO}_3$	Inhibition
	M	M	microliters $\text{CO}_2$	microliters $\text{CO}_2$	per cent
1	$10^{-2}$	$6 \times 10^{-3}$	156	251	38
		$2.5 \times 10^{-2}$	778	788	1
		$7 \times 10^{-2}$	1135	978	0
2	$9 \times 10^{-3}$	$1.55 \times 10^{-2}$	748	696	7
					(13)*
3	$10^{-2}$	$3 \times 10^{-3}$	12	132	91
		$7.6 \times 10^{-3}$	71	321	78
		$1.5 \times 10^{-2}$	530	551	4
4	$1.2 \times 10^{-2}$	$1.2 \times 10^{-2}$	93	390	76
	$1.2 \times 10^{-1}$	$1.2 \times 10^{-1}$	478	680	30
5	$10^{-2}$	$7.6 \times 10^{-3}\dagger$	70	250	72
		$7.6 \times 10^{-2}\ddagger$	>316	470	<33
	$10^{-1}$	$7.6 \times 10^{-3}\dagger$	55	250	78
		$7.6 \times 10^{-2}\ddagger$	78	470	83

\* By colorimetric pyruvate determination.

† Initial concentration.

‡ Final concentration.

The same hypothesis was tested in another way; namely, by allowing bisulfite to act on cocarboxylase at pH about 6 and 28° and then testing for coenzyme activity by adding this mixture to apocarboxylase (alkaline-washed dry yeast) plus pyruvate. In one experiment of this type, 10  $\gamma$  of synthetic cocarboxylase (Merck) were left in contact with  $10^{-1}$  and  $10^{-2}$  M  $\text{NaHSO}_3$  for 74 minutes, after which time the coenzyme-inhibitor mixture was added under nitrogen to the apoenzyme-substrate mixture, which involved a 10-fold dilution of the bisulfite. The result was that the system containing  $10^{-2}$  M  $\text{NaHSO}_3$  (final concentration) showed an inhibition of 63 per cent compared with the bisulfite-free control and with the system

containing  $10^{-3}$  M  $\text{NaHSO}_3$  (final concentration). If the carboxylase inhibition by  $10^{-2}$  M bisulfite, which had been previously observed, had been due to an irreversible splitting of cocarboxylase, marked inhibition

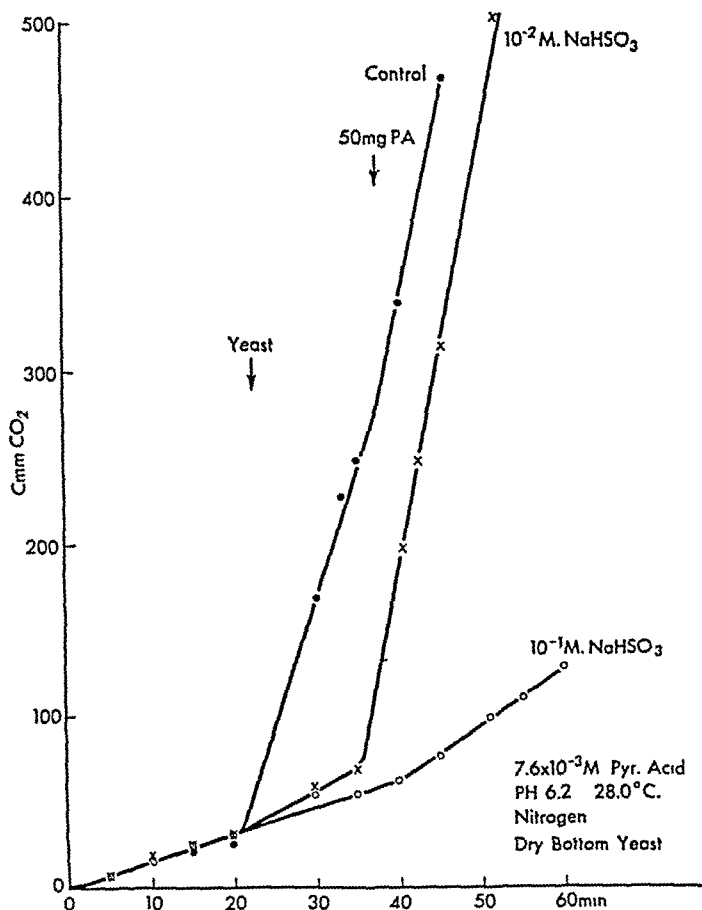


FIG. 4. Relief of bisulfite inhibition of carboxylase by the addition of relatively large amounts of pyruvate.

in the latter system would be expected, because the coenzyme had been in contact for a considerable period with bisulfite of this strength. The absence of any inhibition after diluting to yield a final  $\text{NaHSO}_3$  concentration of  $10^{-3}$  M could be explained either by assuming a complete reversibility

of the coenzyme cleavage or by the more probable assumption that the inhibition by  $10^{-2}$  M bisulfite is not due to a reaction with cocarboxylase.

It now remained to ascertain whether bisulfite damages in an irreversible manner the *protein moiety* of carboxylase. Apococarboxylase was prepared

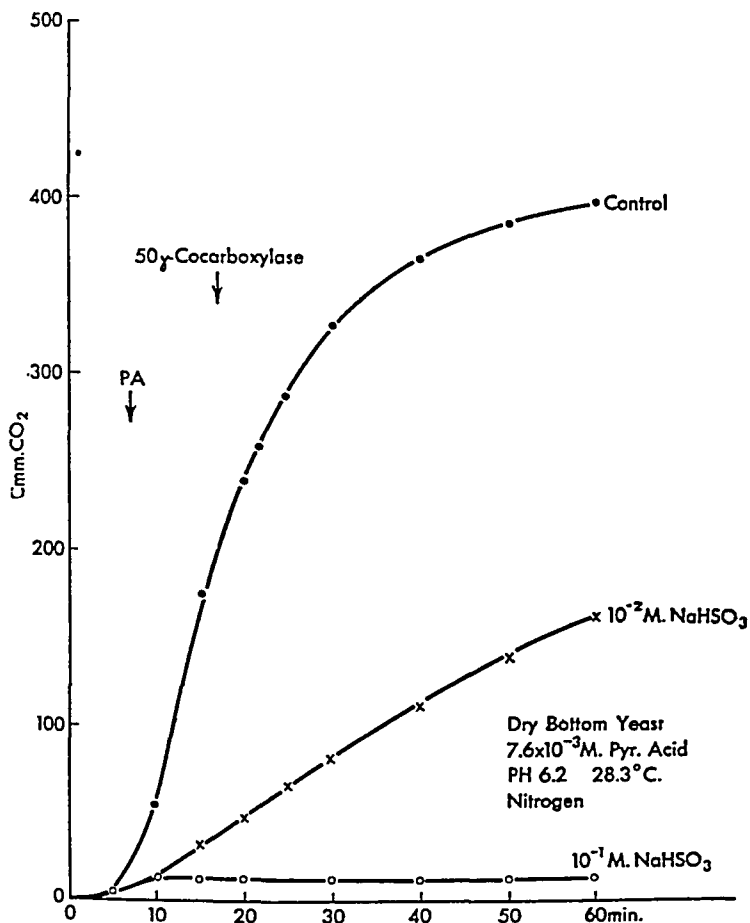


Fig. 5. Failure of cocarboxylase to relieve the bisulfite inhibition of carboxylase

by treating the dry yeast with alkaline phosphate in the usual way (5). After removal of the natural complement of cocarboxylase, the alkali-washed yeast was suspended in a  $10^{-2}$  M bisulfite solution and the suspension was stirred for 20 minutes at 30°, after which it was centrifuged. The sediment was suspended in distilled water and recentrifuged in order to



remove residual bisulfite. It was then suspended in 0.1 M phosphate, pH 6.2, and its apocarboxylase activity was tested manometrically by adding 10, 20, and 30  $\gamma$  of synthetic cocarboxylase. In all three instances an active decarboxylation of pyruvate was observed, increasing somewhat with increasing coenzyme concentration and indicating that  $10^{-2}$  M  $\text{NaHSO}_3$  will not inactivate irreversibly the protein part of carboxylase.

The fact that no component of the diphosphothiamine-magnesium-protein system is irreversibly damaged by bisulfite under the experimental conditions was further substantiated by the demonstration that pretreatment of dry yeast for 45 minutes at  $28^\circ$  with  $10^{-1}$  and  $10^{-2}$  M  $\text{NaHSO}_3$  and subsequent removal of the agent prior to testing for carboxylase activity have no appreciable effect and cause no deficiency in coenzyme. The absence of a *time factor* in the bisulfite effect on the carboxylase activity of dry yeast was ascertained by leaving the dry yeast suspension in contact with  $10^{-2}$  M  $\text{NaHSO}_3$  at  $28^\circ$  for periods varying from 7 to 37 minutes prior to starting the catalysis by adding the pyruvate. The degree of inhibition was approximately the same in all vessels, 77 per cent (7 minutes contact time), 69 per cent (17 minutes), 70 per cent (27 and 37 minutes).

#### *On the Mechanism of Bisulfite Inhibition*

The experimental investigation of the phenomenon of bisulfite inhibition of the carboxylase-pyruvate reaction has established the following facts. The reaction is markedly inhibited by  $10^{-1}$  and  $10^{-2}$  M but not by  $10^{-3}$  M  $\text{NaHSO}_3$ , at pH 6.2 and 4.9, at  $28^\circ$ , and at low pyruvate concentrations ( $7.6 \times 10^{-3}$  M). The inhibition is independent of the state of purity or dispersion of the carboxylase preparation (see Table I); its onset seems to be preceded by a lag period of 5 to 10 minutes after the addition of bisulfite to the enzyme-substrate system. The bisulfite inhibition appears to become more pronounced with time. At constant bisulfite concentration the degree of inhibition decreases with increasing pyruvate concentration and it disappears when the pyruvate is present in excess of the stoichiometric amount required for formation of an equimolar complex with the inhibitor. Upon comparing the rate of reaction with the degree of inhibition at different levels of free pyruvate and pyruvate-bisulfite concentrations, the extent of inhibition is found to decrease as the absolute reaction rate increases with increased substrate and substrate-inhibitor concentration (see Table II). The bisulfite inhibition, once established at low pyruvate concentration, may be relieved by the addition of relatively large amounts of pyruvate, while the addition of synthetic cocarboxylase has no effect. Neither the coenzyme nor the protein moiety of carboxylase appears to be damaged in an irreversible manner by contact with bisulfite

at the pH, temperature, and bisulfite concentrations employed in the kinetic experiments.

The foregoing facts constitute strong evidence that the bisulfite interferes with the enzymatic catalysis by reacting with the substrate to form a pyruvate-bisulfite addition compound (or complex) which is less readily attacked by carboxylase than free pyruvate. Clewing (3) showed many years ago that pyruvic acid combines with secondary alkali sulfites to form very stable double salts or complexes of the formula  $\text{CH}_3\cdot\text{C}(\text{OH})(\text{O}\cdot\text{SO}_2\text{Me})\cdot\text{COOMe}$ , which do not decompose even at  $115\text{--}130^\circ$ . On the basis of the data available it is difficult to decide whether the bisulfite-pyruvate complex is entirely resistant to carboxylase action or whether it is decarboxylated but at a lower rate than free pyruvate. If the former is true, then the slow catalysis occurring at equimolar bisulfite and pyruvate concentration should be a function of the dissociation constant of the complex; *i.e.*, of the amount of free pyruvate in equilibrium with bound pyruvate.

Kerp (4) has measured the dissociation constants of a number of carbonyl-bisulfite complexes. On the assumption that the pyruvate has a dissociation constant of the order of the acetaldehyde-bisulfite complex, *i.e.* of the order of  $k = 2 \times 10^{-6}$ , the equilibrium concentration of free pyruvate in a  $10^{-1}$  M solution of the complex should then be so small as to make it improbable that only the free pyruvate is subject to the attack by the enzyme. This consideration makes it appear likely that the intact pyruvate-bisulfite complex is also decarboxylated by the enzyme but at a lower velocity than the free substrate. In other words, the combination of the pyruvate with bisulfite would tend to decrease its affinity for the enzyme as expressed by its Michaelis constant (*cf.* (8)).

In this connection it is of interest that purified carboxylase is saturated with substrate at  $1.6 \times 10^{-1}$  M pyruvate concentration, as indicated by the maximum rate of the catalysis at pH 6.0 (Green *et al.* (1)). In the present experiments on dry yeast, the highest substrate concentration employed was  $1.2 \times 10^{-1}$  M, at which concentration the pyruvate-bisulfite complex was attacked by the enzyme at about two-thirds the rate at which the free pyruvate was split. It is to be expected that at still higher substrate concentrations the relative inhibition of the pyruvate-bisulfite catalysis approaches zero as the enzyme becomes saturated with the complex. This would correspond to a displacement towards the right of the activity- $P_s$  curve of the carboxylase-pyruvate-bisulfite system as compared with free pyruvate as substrate (*cf.* (8)).

At the substrate concentration at which the bisulfite inhibition was most pronounced ( $7.6 \times 10^{-3}$  M pyruvate) the enzyme was only very incompletely saturated with substrate. Under these conditions the diff

in reaction rate in the control and in the bisulfite-containing systems was most pronounced, as would be expected, on theoretical grounds, of an inhibition of the *competitive* type. The decrease in inhibition upon increasing the substrate concentration at constant inhibitor concentration ( $10^{-2}$  M  $\text{NaHSO}_3$ ), which was observed in this work, is likewise predictable on this basis (*cf.* (8)).

Bisulfite forms also a tight complex with acetaldehyde (4) which was one of the products of the reaction under study. This aldehyde, when formed in appreciable amounts during the catalysis or when added as such to the system, causes an inhibition (5), presumably by affinity for carboxylase. It is to be expected, therefore, that the trapping of acetaldehyde by bisulfite as it arises during the catalysis might relieve the aldehyde inhibition and, under certain conditions, balance the inhibitory effect of bisulfite on the reaction. It is even conceivable that bisulfite, in the presence of a large stoichiometric excess of pyruvate, could activate the catalysis by binding the acetaldehyde, provided that its affinity for the latter is greater than for pyruvic acid.

That, under the experimental conditions chosen here, no appreciable *sulfite cleavage* of cocarboxylase took place may be explained by the fact that the absolute bisulfite concentrations were much lower, and the periods of interaction much shorter, than those employed by Lohmann and Schuster (5) who allowed 2.3 cc. of  $8.3 \times 10^{-1}$  M  $\text{NaHSO}_3$  solution to act, at pH 5, on 130 mg. of cocarboxylase. Under these conditions, the cleavage of the coenzyme was found to be nearly complete after 3 days at room temperature. At the temperature of flowing steam, the reaction goes to completion within 30 minutes (9).

#### DISCUSSION

In the well known fixation process of glycerol fermentation the addition of considerable quantities of sulfites to yeast and sugar, at pH 7 to 8, leads to the formation of equimolar amounts of *glycerol* and aldehyde-bisulfite complex in yields rarely exceeding 60 per cent of the theoretical. The mechanism of this process, as based on our newer knowledge of fermentation (*cf.* (10)), is depicted in Fig. 6. Neuberg and his collaborators, who have done the pioneer work in this field (*cf.* (11)), have maintained in the face of statements to the contrary (12) that the pyruvate-bisulfite complex is as readily fermentable as free pyruvic acid (13). Since pyruvic acid is an intermediate in alcoholic as well as glycerol fermentation (see Fig. 6), it is evident that the sulfite used as steering agent in the latter process does not prevent the *ultimate* decarboxylation of pyruvate by the enzyme carboxylase. The apparent discrepancy between Neuberg's view and the present observations that bisulfite, under certain conditions, inhibits markedly

the action of carboxylase on pyruvate, may be resolved as follows: The experiments by Neuberg and Reinfurth (13) were invariably performed at high pyruvate concentration (0.1 to 0.25 M); i.e., under circumstances in which the inhibition by bisulfite, even when used in equimolar concentration, is relatively small. Furthermore, they were carried out in such a manner that the kinetics of the bisulfite effect could not be determined (incubation of pyruvate-bisulfite mixtures for as long as 7 days at 37° and determination of the aldehyde or CO<sub>2</sub> formed at the end of this period). Their aldehyde yields varied from 46 to 102 per cent of the theory, whereas,

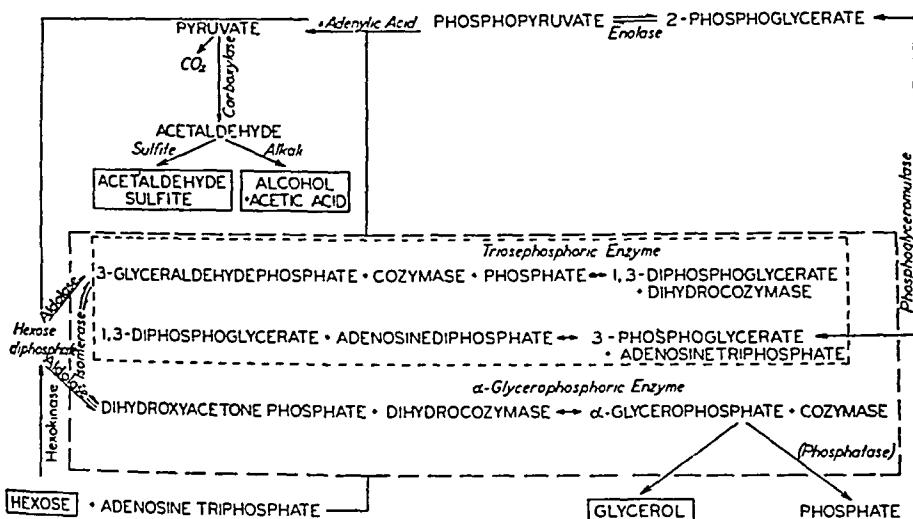


FIG. 6. Detailed schema of glycerol fermentation in presence of sulfite or alkali, based on the work of Neuberg, Embden, Meyerhof, Warburg, and others.

if bisulfite had not interfered with the reaction, the decarboxylation of the pyruvate should have been complete in every instance.

Glycerol is formed during yeast fermentation whenever acetaldehyde is unavailable as hydrogen acceptor to dihydrocozymase. This is the case during the induction period of alcoholic fermentation until sufficient aldehyde has been formed from triose phosphate, in the presence of sulfite and other trapping agents for aldehyde, and in alkaline fermentation in which the aldehyde is changed to alcohol and acetic acid (Fig. 6) or removed by aeration. In industrial practice all of these "abnormal" types of fermentation have certain disadvantages which make it desirable to search for other possibilities of glycerol fermentation. In this connection, attempts to inhibit selectively the acetaldehyde reductase or carboxylase are

of special interest. While in muscle glycolysis pyruvate is the preferred hydrogen acceptor for dihydrocozymase (10), the pyruvic reductase concentration in yeast is too low to compete successfully with the dihydroxyacetone reductase (or  $\alpha$ -glycerophosphoric enzyme) for the reduced diphosphopyridine nucleotide. The advantage of such schemes would be the possibility of *preventing*, by small amounts of inhibitors, either the formation of aldehyde from pyruvate or its reduction to alcohol, in contradistinction to the fixation method which requires quantities of trapping agent equivalent to or larger than the amount of aldehyde accumulating throughout the fermentation. It was with this aim in mind that the effect of bisulfite on carboxylase in low concentration and at acid pH was studied. Cocarboxylase has a structure which is unique among all fermentation catalysts and intermediates; if it could be split by bisulfite into inactive fragments without affecting other components of the system, the fermentation would be switched from the alcoholic to the glycerol type. However, the present experiments show that bisulfite merely slows down the decarboxylation of pyruvate by complex formation, an effect which under the conditions of fermentation would progressively decrease in significance as the amounts of pyruvate formed from phosphopyruvic acid become larger. A search for other inhibitors of carboxylase is therefore warranted (14).

#### SUMMARY

The decarboxylation of pyruvic acid by yeast carboxylase is markedly inhibited by sodium bisulfite, at pH 6.2 and 28°, under conditions in which the inhibitor is present in stoichiometric excess over the substrate or in equimolar amounts at low substrate concentrations.

The bisulfite inhibition appears to be independent of the state of purity or dispersion of the yeast carboxylase preparations used. The inhibition may be relieved by the addition of relatively large amounts of pyruvate, but not by cocarboxylase.

The mechanism of the bisulfite inhibition appears to consist in the formation of a pyruvate-bisulfite complex which is less readily attacked by the enzyme than free pyruvate. The inhibition is therefore of a *competitive* and *reversible* nature.

The bearing of these findings on the mechanism of glycerol fermentation is briefly discussed and it is pointed out that an inhibitor of a different type than bisulfite would be more suitable for changing the yeast fermentation from alcohol to glycerol production.

The authors are indebted to Dr. Carl Neuberg, Dr. Otto Meyerhof, Dr. Dean Burk, and Dr. Eduard Farber for stimulating discussions of the problem.

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# ON EXTRACTS OF CARP SPLEEN AND THEIR EFFECT ON CARBOXYLASE AND COCARBOXYLASE

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Studies on the mechanism of glycerol fermentation in this laboratory have led to the demonstration that yeast carboxylase may be inhibited, under certain conditions, by bisulfite (1). When it became desirable to employ inhibitors of a greater specificity than bisulfite, Dr. S. Ochoa directed our attention to the recent work on the inactivation of thiamine by fish tissue (2, 3). Inasmuch as carboxylase is a *diphosphothiamine-magnesium-protein* (4, 5), it seems possible and indeed probable that co-carboxylase (thiamine diphosphate) is attacked by the enzymatic principle, present in fresh carp tissues, in a manner analogous to thiamine. The present experiments strengthen this expectation.

## EXPERIMENTAL

### *Materials and Methods*

Carp spleen was chosen as the source material for the present experiments since Sealock *et al.* (3) have shown that this tissue has the highest content of the thiamine-splitting principle among the carp tissues examined. In order to obtain uniform and reproducible results, a number of fresh carp spleens were converted into a dry, stable powder by the acetone treatment described in detail by these authors. This acetone-dried material was used for the preparation of all the carp spleen extracts employed in this work. Prior to each experiment, varying amounts of the spleen powder were suspended in 10 to 20 volumes of 10 per cent NaCl solution by grinding in a mortar, and the insoluble residue was removed by centrifuging at 3500 R.P.M. The supernatant extract, containing the active principle, was reddish and somewhat opalescent. Upon storage of such saline extracts in the refrigerator the activity towards thiamine was preserved for periods of a few days.

The activity of the saline carp spleen powder extracts towards thiamine was ascertained by a photoelectric method based on the diazo reaction of Prebluda and McCollum, and representing a modification<sup>1</sup> of the procedure

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<sup>1</sup> The kindness of Dr. D. Melnick in placing the details of this modified method at our disposal is gratefully acknowledged. Owing to the absence of interfering pigments, the filtration of the reaction mixtures through synthetic zeolite could be omitted in the present experiments.



described by Melnick and Field (6). The amount of thiamine remaining intact after incubation with the carp spleen extract at 37° and pH 7.4 was determined by measuring the light absorption of the xylene extract of the pink diazo compound formed with the reagent in a Klett-Summerson photoelectric colorimeter with a light filter having preferential transmission at 420 m $\mu$ . A calibration curve was constructed with the aid of a series of thiamine standards.

In the experiments dealing with the effect of the carp spleen extracts on yeast carboxylase and cocarboxylase, the enzyme or coenzyme activity remaining after incubation with the spleen extract was determined manometrically in the usual manner (7, 8). In a few instances, the results of the manometric assay were controlled by pyruvic acid determinations by the colorimetric method of Friedemann and Haugen (9).

The nitrogen content of the tissue extracts was assayed by the Pregl micro modification of the Kjeldahl method.

The electrophoretic experiments were performed with the Tiselius apparatus as manufactured by the Klett Manufacturing Company, Inc., of New York.

#### *Some Properties of Carp Spleen Extracts*

The content in total nitrogen of the saline extracts prepared from acetone-dried carp spleen powder was 1.5 mg. per cc. on the average. At pH 7.4 and 37°, 1 cc. of spleen extract would split about 37  $\gamma$  of thiamine hydrochloride in 1 hour, as determined by the photoelectric method referred to above.

A preliminary examination of such carp spleen extracts in the *Tiselius electrophoresis apparatus* had the following results. A dialyzed sample of spleen extract was placed in the tall section analytical cell of Tiselius (see Longworth (10)) and subjected to electrolysis at 1.0° for 11,950 seconds at a potential gradient of 3.09 volts per cm.; a 0.05 M phosphate buffer of pH 7.7 and 0.12 ionic strength was employed as the supernatant solution. Visual observation and photographic records obtained with the Svensson crossed slit and the Longworth schlieren scanning technique disclosed the presence of three components of different electrochemical behavior (see Fig. 1). The component present in the highest relative concentration exhibited an anodic mobility of  $17.6 \times 10^{-5}$  cm. per volt per second in the ascending limb of the apparatus and of  $16.5 \times 10^{-5}$  in the descending limb. Of the remaining two components, one showed an anodic mobility of  $5.6 \times 10^{-5}$  cm. per volt per second in the ascending and of  $4.95 \times 10^{-5}$  in the descending limb, while the third component was stationary throughout.

With a view to securing some information on the mobility of the thiamine-splitting principle present in these extracts, an experiment was performed

with the Tiselius separation cell of 11 cc. capacity. The components of highest and of zero mobility were trapped in the left upper and right upper compartments of the cell respectively, and the contents of the various compartments were analyzed for total nitrogen content and their enzymatic

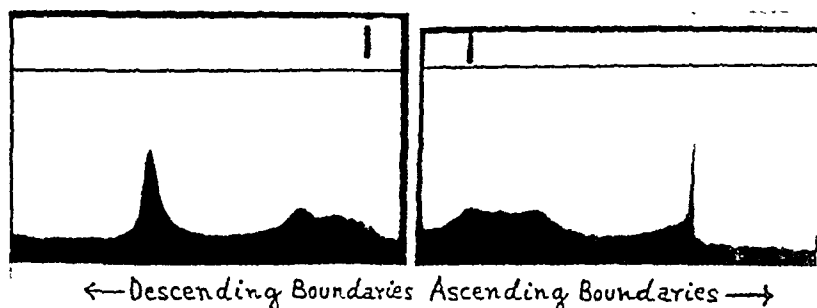


FIG. 1. Electrophoresis diagram obtained from crude carp spleen extract. For experimental conditions see the text.

TABLE I

*Assay of Fractions Obtained by Electrophoretic Separation of Carp Spleen Extract*

2 gm. of carp spleen acetone powder extracted with 20 cc. of 0.05 M phosphate buffer, pH 7.76, at low temperature for 18 hours; suspension centrifuged 20 minutes at 3300 R.P.M., supernatant solution ultracentrifuged for 10 minutes at 15,000 R.P.M.; clear, reddish supernatant fluid used for experiment without dialysis.

16,740 seconds electrolysis at potential gradient  $F = 3.5$  volt per cm.,  $1^\circ$  and pH 7.7 (0.05 M phosphate buffer); no compensation.

Limb of Tiselius cell	Compartment	Fraction present	Total nitrogen <i>mg. per cc.</i>	Enzymatic activity towards thiamine <i><math>\gamma</math> split per cc. per hr.</i>
Anode	Left upper	Fast component	0.58	3.5
	" lower	Mixture	1.31	78.4
	Bottom	"	3.98	97.1
Cathode	Right lower	"	4.26	87.6
	" upper	Stationary component	3.2	10.1

activity towards thiamine. In order to obtain as much material in solution as possible, a ratio of spleen powder to buffer solution of 1:10 was employed in this experiment. The results of the assays performed on the different fractions are summarized in Table I. They seem to indicate that the enzymatic activity is associated neither with the highly mobile nor with the stationary component but that it is probably linked to material of inter-

mediate mobility. In order to avoid the loss of activator or coenzyme occurring during dialysis (*cf.* (2)), the extract was not dialyzed prior to the experiment.

### *Effect of Carp Spleen Extracts on Carboxylase and Cocarboxylase*

Yeast maceration extract (Lebedev juice), upon incubation with carp spleen extract at 37°, loses its power to decarboxylate pyruvate. The maceration juice was prepared by suspending dried Krueger's bottom yeast in 3 parts of tap water, incubation for 90 minutes at 37°, and centrifuging for 15 minutes at 3400 R.P.M. 2 cc. of the supernatant, fairly clear extract were mixed with 1 cc. of 0.1 M phosphate buffer of varying pH and with 1 cc. of carp spleen extract. After the reaction mixtures were incubated for 1 hour at 37°, 1 cc. was placed in the main compartment of conical Warburg vessels. The pH was shifted to about 6 by the addition of 2 cc. of 0.1 M secondary phosphate solution. The side bulb of the vessels contained 0.3 cc. of sodium or lithium pyruvate solution, corresponding to 2.2 to 4.5 mg. of free pyruvic acid. After the vessels were equilibrated at 28° against air as the gas phase, the substrate was added to the enzyme preparation and the changes in gas pressure were recorded in 5 minute intervals for 45 to 50 minutes with simple Warburg-Barcroft manometers. Suitable controls were run, 10 per cent NaCl solution being substituted for the carp spleen extract. It was found in this way that the treatment of the yeast extract with the carp spleen extract resulted in an inhibition of its carboxylase activity ranging from 66 to 90 per cent, irrespective of whether the incubation was carried out at pH 6.6, 7.1, or 7.9. An increase of the pyruvate concentration from 4.9 to 12 and 24 mg. in terms of pyruvic acid as well as the addition of 50  $\gamma$  of synthetic cocarboxylase (Merck) failed to relieve the inhibition produced by the preceding treatment of carp spleen extract at pH 6.6, indicating that it is of a non-competitive character. By the way of example, the result of a manometric carboxylase assay on a carp spleen-treated yeast maceration extract is reproduced graphically in Fig. 2. The inhibition of carboxylase activity was confirmed by colorimetric determination of the pyruvate remaining in the system at the end of the experiment. In these experiments, the carboxylase was present in *crude, dissolved* form. It was of interest to examine the effect of carp spleen extract on *purified* carboxylase *in solution* on the one hand and on a *dry yeast* suspension, in which enzyme is present in *undissolved* form, on the other hand. To this end, yeast carboxylase was purified according to the method of Green *et al.* (4) by ammonium sulfate fractionation of yeast maceration extract, up to Step 4. After the purified enzyme was incubated with carp spleen extract at pH 6.5, the activity of the enzyme preparation, when tested manometrically against 5.0 and 50 mg. of pyruvic acid

in citrate buffer of pH 6.05, was found to be practically abolished. In order to test the action of the fish tissue principle on carboxylase as a constituent of yeast cells the membranes of which have been ruptured by autolytic drying, 100 mg. of dried Krueger's bottom yeast were suspended in 5 cc. of 0.15 M phosphate buffer of pH 6.58. After addition of 3 cc. of carp spleen extract and 2 cc. of additional phosphate buffer, the mixture was incubated for 93 minutes at 37°. A control mixture, containing 10 per cent NaCl solution instead of the spleen extract, was treated in the

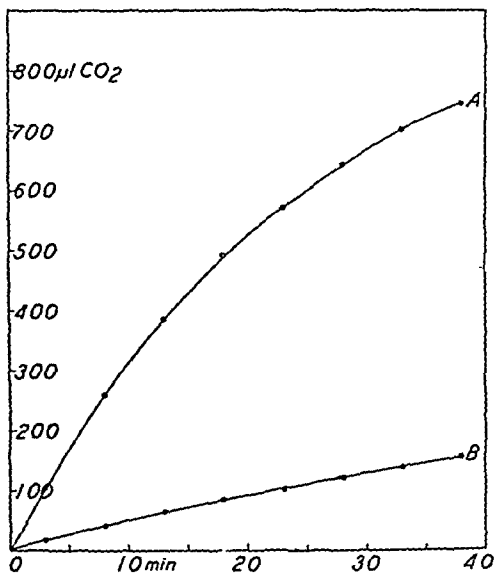


FIG. 2. Inhibition of yeast carboxylase by carp spleen extract. Curve A, rate of pyruvate decarboxylation by control; Curve B, activity of Lebedev juice after treatment with carp spleen extract for 1 hour at pH 7 and 37°.

same way. The manometric assay of both mixtures showed that an inhibition of 41 per cent had occurred in the system containing the carp spleen extract. The addition of 50  $\gamma$  of synthetic cocarboxylase failed to relieve the inhibition. It remained to be determined whether the attack of the carp spleen principle is directed towards the prosthetic group, *i.e.* cocarboxylase, or the protein moiety of the yeast enzyme. Two sets of experiments were performed to test the effect of carp spleen extracts on dry yeast which had been freed of cocarboxylase by treatment with alkaline phosphate (apocarboxylase) and on synthetic diphosphothiamine (cocarboxylase). The apocarboxylase was prepared by treating 1 gm. of dried

Krueger's bottom yeast twice with 50 cc. of 0.1 M  $\text{Na}_2\text{HPO}_4$  solution for 12 minutes at  $30^\circ$  and washing the residue briefly with distilled water, centrifuging for separation of the solid material. This treatment removes the coenzyme from the undissolved protein component and leaves the latter in a state in which it may readily be reactivated by the addition of synthetic or natural cocarboxylase plus magnesium ions (7, 8). The apoccarboxylase was then suspended in 10 cc. of phosphate buffer, pH 6.5. 4 cc. of this suspension were incubated with 2 cc. of carp spleen extract for 2 hours at  $37^\circ$ . In order to remove the fish principle prior to the mano-

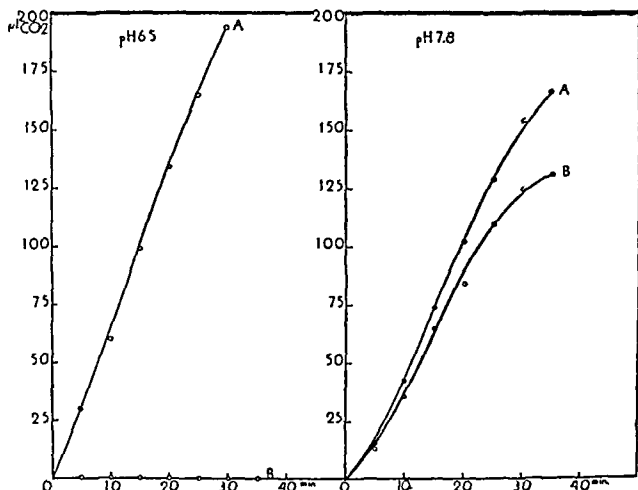


FIG. 3. Destruction of cocarboxylase by carp spleen extract. Left-hand diagram, Curve A, rate of pyruvate decarboxylation by control; Curve B, activity of cocarboxylase after incubation with carp spleen extract for 2 hours at pH 6.5 and  $37.5^\circ$ . Right-hand diagram, coenzyme incubated with carp spleen extract at pH 7.8.

metric carboxylase determination, the incubated mixture was centrifuged, the residue was suspended in 10 cc. of 0.1 M phosphate buffer, pH 6.2, again centrifuged, and resuspended in the same buffer to make a final volume of 5 cc. As usual a control was run, 10 per cent. NaCl solution being substituted for the spleen extract. Both preparations were tested for their apoccarboxylase activity by the addition of synthetic cocarboxylase, lithium pyruvate, and magnesium chloride. The rate of liberation of  $\text{CO}_2$ , i.e. of decarboxylation of the pyruvate, was the same in both cases. The absolute activity was rather low in this experiment, probably because of the well known instability of the apoccarboxylase preparation.

The most clear cut results were obtained upon treating synthetic co-

carboxylase with carp spleen extract and adding the mixture to alkaline-washed dry yeast in order to ascertain the effect of the fish principle on the thiamine pyrophosphate. 100  $\gamma$  of synthetic cocarboxylase (Merck) dissolved in 1 cc. of water were incubated for 2 hours at 37° with 0.5 cc. of carp spleen extract and 0.5 cc. of 0.1 M phosphate buffer of varying pH, and then an aliquot of these mixtures, corresponding to an original amount of 15  $\gamma$  of coenzyme, was added to a suspension of 100 mg. of alkaline-washed

TABLE II  
*Action of Carp Spleen Extract on Carboxylase and Cocarboxylase*

Experiment No.	Material	pH*	Manometric assay				
			Pyruvic acid	Cocarboxylase	Experimental mixture	Control mixture	Inhibition of activity
			mg.	$\gamma$	microliters CO <sub>2</sub>	microliters CO <sub>2</sub>	per cent
1	Lebedev juice	7.1	2.2		71	215	67
2	" "	7.1	4.5		157	745	79†
3	" "	6.6	4		19	131	85
		7.9	4		16	133	88
4	" "	6.6	4.9	50	37	530	78
			12	50	63	678	91
			24	50	48	750	94
5	Dry yeast	6.6	4	50	130	220	41
6	Purified carboxylase	6.5	50		5	367	99
7	Cocarboxylase	6.6	5		0	207	100
		6.6	5		0	194	100
		7.8	5		131	166	21
8	"	6.6	5		12	235	95
		6.6	5		9	204	96
		7.8	5		151	156	3
9	Apocarboxylase	~7	5	50	33	36	

\* During incubation with carp spleen extract. The pH during the manometric assay was always close to 6.0.

† Chemical analysis of the pyruvate present before and after the experiment in the manometer vessels yielded a value for the extent of inhibition of 74 per cent.

dried Krueger's bottom yeast (apocarboxylase) and 5 mg. of pyruvic acid (as lithium salt) plus 0.1 mg. of magnesium (as chloride) in a total volume of 3 cc. of phosphate, pH 6.0, and the rate of CO<sub>2</sub> evolution was observed manometrically for about 50 minutes at 30°. The result of these experiments was that incubation of cocarboxylase with carp spleen extract at pH 6.4 to 6.75 under the conditions mentioned above produces complete inhibition or destruction of the coenzyme, whereas a similar treatment at pH 7.8 leads to little or no loss of activity (Fig. 3). Again, appropriate controls were run with NaCl solution instead of the fish tissue extract.

The results of the experiments discussed in this section are summarized in Table II.

#### DISCUSSION

The inhibition of yeast carboxylase and the destruction of cocarboxylase by saline extracts of acetone-dried carp spleen tissue appear to be established as an experimental fact by the data presented in this paper. While it is tempting to identify the agent responsible for this phenomenon with the thiamine-destroying principle (*Chastek paralysis factor*) known to be present in such fish tissue extracts, proof for this assumption is as yet lacking. This would require the demonstration that upon fractionation of the active spleen or other fish tissue extracts by different methods the ability of the various fractions to split the thiamine runs strictly parallel to their inhibitory effect on carboxylase. The same reservation holds with regard to the mechanism of the inhibition beyond the statement that it is non-competitive in character with respect to the substrate of carboxylase, pyruvic acid. A cleavage of cocarboxylase analogous to that of thiamine by the Chastek factor (2) would be expected to yield 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-hydroxyethylidiphosphothiazole. The actual isolation and identification of these split-products, if formed under the action of the carp spleen principle, would require much larger amounts of cocarboxylase than are available to the present authors. The failure of synthetic cocarboxylase to restore the carboxylase activity of yeast maceration extract and of a dry yeast suspension after treatment with carp spleen extract might be explained by assuming that the pyrimidine portion of the coenzyme has been split off, leaving the diphosphothiazole portion combined with the enzymatic protein and thus preventing the combination of the latter with fresh coenzyme molecules. It is of interest to note that free cocarboxylase is more readily attacked by the carp spleen agent at pH 6.5 than at pH 7.8, while thiamine is split with increasing rapidity at increasingly alkaline pH values, reaching its optimum at pH 9.1 (Sealock *et al.* (3)). It is possible, and indeed probable, that the presence of the acidic pyrophosphate radical in cocarboxylase is responsible for this shift in the activity-pH optimum of the reaction, since it is well known that the position of the latter is a function not only of the enzyme but also of the structure of the substrate (*cf.* Haldane and Stern (11)).

Various mammalian tissues, *e.g.* kidney and intestinal mucosa, contain a phosphatase which decomposes cocarboxylase into free thiamine and phosphate. The inhibitory effect of carp spleen extracts on carboxylase and cocarboxylase could, therefore, also be interpreted in terms of such a phosphatase action, which would occur independently of the cleavage of free thiamine, were it not for the fact that the addition of synthetic co-

carboxylase failed to relieve the inhibition produced by the carp spleen extracts.

Aside from their interest for the problem of glycerol fermentation (*cf.* (1)), the present observations on the effect of fish tissue preparations on carboxylase suggest the possible use of the carp spleen enzyme as a tool for the differentiation between thiamino- and pyridino- or flavoproteins. In interpreting results obtained by the action of carp spleen extracts on complex biological systems, it should be borne in mind that at pH 6.5 free cocarboxylase appears to be more sensitive to the agent than its combination with the enzymatic protein (carboxylase) and also that the sensitivity of the latter is enhanced when it is brought into solution and when it is chemically purified.

#### SUMMARY

Yeast carboxylase in different states and degrees of purity is appreciably inhibited by treatment with saline carp spleen extracts containing the thiamine-destroying Chastek paralysis factor.

Free, synthetic cocarboxylase is likewise inactivated by carp spleen preparations at pH 6.5 but not appreciably at pH 7.8.

The preliminary examination of carp spleen extract in the Tiselius electrophoresis apparatus has disclosed the presence of three components differing in their electrochemical properties.

The authors wish to thank Mr. James S. Wallerstein, President of the Overly Biochemical Research Foundation, for his interest which made this investigation possible.

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# THE TEMPERATURE COEFFICIENT FOR THE DENATURATION OF CHORIONIC GONADOTROPIN BY UREA

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The velocity constant for the first order reaction, which governs the denaturation of the chorionic gonadotropin by urea to a product with 8 per cent of the original biologic activity, was recently established at  $37.5^{\circ}$  (1). The present report is concerned with the temperature coefficient of this reaction, which was studied at increments in the temperature range  $24.9$ – $46.1^{\circ}$ .

The details of the denaturation and of the biologic assay have been described (1). At the higher temperature range in the present studies the inactivation progresses so rapidly that the time (about 1.5 minutes) necessary to establish equilibrium of temperature would introduce an error in the determination if the velocity constant were calculated from zero time. Since, however, a first order reaction need not be calculated from zero time, the bioassay was performed on a sample immersed in the bath after a 5 minute interval and then on samples removed at 10 minute intervals. The reaction was stopped by dilution with water and cooling. The assay data and velocity constants derived from these data are given in Table I.

In Fig. 1 are charted the values (obtained from Table I) of the logarithm of the velocity constant against  $1/T$ , where  $T$  is the absolute temperature. The result is clearly, within the accuracy of the bioassay, a straight line, showing that

$$\log k = C - a/T \quad (I)$$

Differentiating this simple Equation I, and finally integrating, Arrhenius developed the well known equation,

$$Q = R2.3 \frac{(\log k_2 - \log k_1)}{(1/T_1 - 1/T_2)} \quad (II)$$

in which  $k_1$  and  $k_2$  are the rates at the absolute temperatures  $T_1$  and  $T_2$ ,  $R$  is the gas constant in calories, and  $Q$  is the activation energy for the reaction in calories per mole.

If the velocity constants (see Table I and Fig. 1) 0.15 and 0.0072 for  $46.1^{\circ}$  and  $24.9^{\circ}$ , respectively, are used,  $Q$  becomes  $27,100 \pm 1000$  calories per mole in Equation II.

TABLE I

*Inactivation of Chorionic Gonadotropin (Prolan) by Exposure to 40 Per Cent\* Aqueous Urea Solution at Various Temperatures*

Temperature of inactivation °C.	Time of inactivation min.	Ratio of control to experimental dose	Mean uterine weight†		Estimated recovery of hormone per cent	Correct assay, range 19 of 20 times per cent	<i>k</i> (min. <sup>-1</sup> )‡
			Control mg.	Experimental mg.			
46.1	5.0	0.5		40 ± 4.5	38	±4.5	0.14
	10.0	0.25		54 ± 4.5	23	±2	
46.1	5.0	0.5	60 ± 9	55 ± 8	48	±7	0.15§
	10.0	0.25	53 ± 7	72 ± 13	27	±8	
44.9	5.0	0.5	53 ± 8	46 ± 7	47	±11	0.12
	10.0	0.25	53 ± 8	51 ± 8	29	±4	
44.9	20.0	0.2	74 ± 6	40 ± 5	13	±3	0.14
37.5							0.036
31.9	60.0	0.5	80 ± 7	29 ± 2	24	±5	0.028
	120.0	0.25	80 ± 7	61 ± 8	18	±2	0.019
24.9	60.0	1.0	57 ± 8	34 ± 5	68	±16	0.0072
	180.0	0.5	59 ± 8	33 ± 6	33	±8	0.0073

\* 40 parts of urea, 60 parts of H<sub>2</sub>O, 0.6 part of NaCl, 0.4 part of prolan, pH 7.0.

† Mean plus standard deviation of the mean.

‡ Velocity constant.

§ Solution without 0.6 per cent NaCl.

|| Data from previous publication (1).

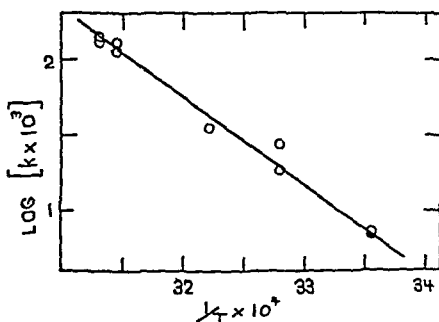


FIG. 1. The logarithm of the velocity constant is plotted against the reciprocal of the absolute temperature, the results of which indicate a straight line function. This line may obviously be used as a regression line to calculate the standard error of the Arrhenius constant. The line was rotated by a distance at each extreme temperature equal to the standard deviation of the mean of the distances of the points to the line. *Q* was then recalculated.

With substitution of 27,100 calories for *Q* and 0.0072 for *k* in Equation II, *k* at 0° becomes 0.000111 or, converted to seconds,  $1.85 \times 10^{-6}$ .

With the above data, the entropy of activation which entails the de-

naturation may be calculated from Equation III for the absolute rate of denaturation (2) which at 0° becomes

$$k_0 = 5.7 \times 10^{14} \times e^{-Q/RT} \times e^{\Delta S/R} \quad (\text{III})$$

$\Delta S$ , the entropy of activation, is 14.1 calories degree<sup>-1</sup> mole<sup>-1</sup>.

The temperature coefficient for a 10° interval becomes (0.15/0.0072)<sup>10/21.2</sup> which equals 4.2.

#### DISCUSSION

The studies of the rates of inactivation of chorionic gonadotropin under various conditions (1, 3) have revealed that there are a number of distinct reaction products characterized by less biologic activity than the intact hormone. The reaction in 40 per cent urea solution at 37.5° yields a reaction product with 8 per cent of the original activity, which slowly goes over to a form retaining 4 per cent of the original activity; a product with 4 per cent biologic activity is also obtained by heat inactivation at 100°. At room temperature in aqueous solution a product possessing about 20 per cent of the original activity is obtained. The calculated results of the present study are based on the assumption that the product formed at 37.5° is also formed in the range of temperature studied. The correctness of this assumption is substantiated by the results of Fig. 1, the data of which are based on the assumption and upon the agreement of the velocity constants calculated at different time intervals for the same temperature.

The heat of activation of 27,100 calories per mole, with a temperature coefficient of 4.2 for chorionic gonadotropin in urea solution, agrees almost exactly with that of the hydrolysis of cane sugar in water, which is pseudo monomolecular. The values are obviously unique for a denaturation, but the denaturation is in itself unique in that it represents only a part of the final process. This is contrary to the conception based on heat denaturation with concomitant precipitation that "what is peculiar about denaturation as a complex reaction is that it is an all or none reaction" (4). Although the denaturation of chorionic gonadotropin is exceptional with respect to both heat of activation and entropy of activation, it is comparable with other denaturations when compared on the basis of free energy of activation. The value estimated from the data by substitution in Equation IV is about 23,000 calories mole<sup>-1</sup> at 36°, which agrees well with that for hemoglobin, which is 28,000 calories mole<sup>-1</sup>.

$$\Delta F = \Delta H - T\Delta S \quad (\text{IV})$$

where  $\Delta H$  is the heat of activation,  $\Delta F$  is the change in free energy, and  $\Delta S$  is the change in entropy.

While considerably more study of the denaturation of the gonadotropin is warranted, in the light of the results at hand one can conceive of the

dissociation of denaturation as taking place in steps, each step producing a more dissociated molecule of lesser biologic activity. Since no molecule other than water or urea could enter into the reaction and since it is difficult to picture these substances inducing structural changes (in the sense of atomic linkage), this concept must lead to the deduction that the biologic activity is dependent upon the spacing of active points (secondary valence foci) on the surface of the molecule. Structurally the active points are still present in the dissociated molecule, but they become improperly spaced and so lead to lesser activity. In this respect the biologic activity of the hormone is analogous to a contact catalyst (5) in which the spacing of the active points functions to determine not only the degree but also the qualitative nature of the response.

#### SUMMARY

The inactivation rate of chorionic gonadotropin in aqueous 40 per cent urea solution to a product with 8 per cent of the original biologic activity was studied for the temperature range 46.1–24.9°, with the following results.

The velocity constant,  $k$  ( $\text{min.}^{-1}$ ), was 0.15 at 46.1° and 0.0072 at 24.9°.

The heat of activation for the denaturation was 27,100 calories  $\text{mole}^{-1}$  and the entropy of activation for the denaturation was 14.1 calories  $\text{degree}^{-1} \text{mole}^{-1}$ .

The temperature coefficient for a 10° increment was 4.2.

Although the denaturation is exceptional with respect to both the heat of activation and entropy of activation, it is comparable with other denaturations when compared on the basis of free energy of activation.

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# CONTENT AND STATE OF GLUTATHIONE IN THE TISSUES OF THE EYE\*

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Data on the content and state of glutathione in the tissues of the eye are scarce (1-3). A more extensive study seemed desirable since the tissues of the eye differ widely in respect to their origin and their function, and because of the possible rôle of glutathione as a component of the oxidation-reduction system, the activity of which seems to be essential for the secretion of the intraocular fluid (4).

## *Technique*

The bulk of the material which we examined came from bovine eyes. They arrived at the laboratory about 3 hours after the death of the animal and were used immediately. The lapse of time between killing and arrival at the laboratory, as well as the handling of the eyes in the slaughter-house, seemed to be without influence on the glutathione level, since essentially the same figures were obtained with extracts which were prepared in the slaughter-house immediately after the animal was killed.

The method of isolation of the ciliary processes has been described elsewhere (5). For samples of lens cortex we used the outer layer, which is most easily teased off, while the samples of lens nucleus consisted of only the innermost part of the lens. The intermediate portions were discarded in these experiments. The corneal epithelium was scraped off with a scalpel. A complete separation is possible because of the smooth surface and toughness of the stroma. In the experiments with retina we took the whole retina except for the pigment epithelium. The dissection of the other tissues of the eye does not require special comment.

The data in the literature on the glutathione content of tissues are subject to considerable uncertainty. In many tissues this level varies over a wide range and many determinations have to be carried out in order to obtain representative figures. A comparison of the available results is rendered difficult because entirely different methods are used for the determination of glutathione, without sufficient assurance that the frequent discrepancies encountered are not merely of a technical nature. This seems to hold particularly when different methods are also employed for the

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reduction of oxidized glutathione. We have tried to avoid this objection by using two different methods for determination and reduction of the glutathione in the tissues studied. We have compared (Table II) the glyoxalase method, as elaborated by Woodward (6) and the reduction procedure of Dohan and Woodward (7) with a colorimetric method, based on the nitroprusside test in the modification proposed by Fujita and Numata (8), and the reduction technique devised by the same authors (9).

*Glyoxalase Method*—We adopted Behrens' modification of Woodward's original method for the preparation of glyoxalase, using the same strain (Standard Brands No. 189) as the starting material, since higher activities were obtained by this procedure (10). Considerably higher activities claimed by Woodward (6) are apparently obtained only with exceptional yeast strains (personal communication).

Methylglyoxal was prepared according to Henze and Müller (11). The tissues were extracted with amounts of 2.3 per cent sulfosalicylic acid which provided suitable dilutions for the determinations. The samples were ground in a porcelain mortar without addition of sand. Extraction with 2 per cent metaphosphoric acid gave higher blanks and lower activities. The determinations were carried out manometrically. 0.5 ml. of a 15

20 per cent suspension in distilled water of the glyoxalase preparation was pipetted into the main compartment of each Warburg flask. The concentration was adjusted within the indicated limits so as to give highest activity and the least disturbing blank. The suspensions were freshly prepared for each series, although no decrease of the activity was encountered if they remained in the ice box for 2 days. 0.2 ml. of a 1 per cent solution of methylglyoxal and 0.2 ml. of a 0.2 M solution of sodium bicarbonate were likewise placed in the main compartment. In order to obtain optimal activities with different yeast preparations, a small adjustment (0.2 to 0.4 ml.) of the amount of added bicarbonate usually is required. 0.5 ml. of the tissue extracts previously neutralized with a small amount of saturated sodium carbonate is placed in the side arm. Three standard samples covering the range from 0.025 to 0.15 mg. of glutathione dissolved in 0.5 ml. of 2.3 per cent sulfosalicylic acid and a blank were carried along in each run. The vessels were flushed with a mixture of 95 per cent  $N_2$  + 5 per cent  $CO_2$  for 10 minutes. The contents of the side arms were dipped over, followed by an additional shaking period of 5 minutes for equilibration. The temperature of the water bath was 30°. Readings were taken for 20 minutes at 5 minute intervals. The  $CO_2$  output for 0.1 mg. of glutathione for 20 minutes, corrected for the blank, was approximately 97 c.mm.

*Colorimetric Method*—The colorimetric determination, based on the nitroprusside reaction, was carried out essentially according to the pro-

cedure of Fujita and Numata (9). Metaphosphoric acid was used as extracting fluid and the grinding of the tissue was again carried out in the absence of sand. In order to facilitate thorough mixing of the reagents, the extract and reagents were pipetted into wide 50 ml. centrifuge tubes. Ammonia was added, the sample was thoroughly mixed, and readings were taken within 1 minute after the addition of the ammonia, with a Klett-Summerson photoelectric colorimeter and a No. 54 filter. Standards and blank were run simultaneously with each set of samples. With sufficient time for thermoequilibration of all samples, temperature corrections became unnecessary.

Testing the influence of various factors on the color development, we found that a lapse of time up to 2 hours between preparation and use of the nitroprusside solution and also between addition of the fresh nitroprusside solution and of the ammonia to the sample was without an appreciable effect. Further delay gave deviating results. The presence of a considerable amount of  $H_2S$  in the sample did not interfere with the color development. Although sufficiently reproducible readings were obtained when taken within 1 minute after the addition of the ammonia, we tried to find means to prevent the well known fading of the color. Abderhalden and Wertheimer (12) observed a decrease of the fading rate on addition of cyanide and we were able to confirm this finding qualitatively. Assuming that the disappearance of color might be catalyzed by traces of metals, we added various metal binders: sodium pyrophosphate, potassium ethyl xanthate, sodium citrate, allylthiourea, sodium diethyldithio carbamate, and hydroxyquinoline. A stabilizing effect was observed only with the last. 2 mg. of hydroxyquinoline inhibited the fading of the color in a 0.1 mg. GSH<sup>1</sup> sample about 50 per cent. The rate of fading was not altered by the addition of copper or by development of the color *in vacuo*. These data make it seem improbable that the fading is due to an oxidation or to other metal-catalyzed reactions. We were unable to find in the literature on the reaction mechanism of the nitroprusside reaction an explanation for the effect of hydroxyquinoline.

In reduction of the extracts the original prescriptions of the authors were followed closely. In most instances sulfosalicylic acid extracts were also used for reduction with  $H_2S$ , since such extracts could be used for the manometric as well as for the colorimetric determination, and tests with known amounts of oxidized glutathione showed satisfactory recoveries. In several experiments (Table II) Fujita and Numata's method was used for the reduction of the tissue itself. In this case the reagents were added not to the extract but to the tissue suspension which contained 0.5 gm. of ground tissue in a volume of 5 ml.

<sup>1</sup> Glutathione.



The incubation of tissue preparations with oxidized or reduced glutathione was carried out under aerobic and anaerobic conditions. The tissue (400 to 500 mg.) was minced with scissors and pipetted into 50 ml. Erlenmeyer flasks for aerobic experiments and into Thunberg tubes when anaerobiosis was required. The tubes were evacuated with a Cenco Hyvac pump for 10 minutes. Some difficulty was encountered in the case of the lens, which foamed badly. The vessels were immersed in a water bath of a Warburg apparatus, maintained at 37.5°, and agitated by the attached shaker device. The oxidized glutathione was prepared by bubbling oxygen through a solution of reduced glutathione adjusted to pH 8. A sample of cozymase which was used in one of the experiments was prepared (Step B) according to Meyerhof and Ohlmeyer (13) and tested for activity in a lactic dehydrogenase system.

### Results

*Content*—In order to ascertain the comparability of our results, we tested the glutathione content in some organs which had been examined by other authors. From the data summarized in Table I there appears to be a point of general agreement in the fact that the figures of different authors for certain organs are scattered over a wide range but in other instances remain within narrow limits. From this it would seem that the range of the values is just as characteristic for the respective organs as the average values themselves and should be adopted as the preferred notation. This would conform to Ennor's (14) conclusions which were based upon the variability of the content of glutathione in liver. As far as it is possible to make a comparison in view of the scattering of the results, our figures agree with those of the other authors in respect to reduced glutathione. Some discrepancy was observed in the case of rabbit blood, for which Dohan and Woodward (7) found a content of 49 mg. per cent, whereas our highest value is 36 mg. per cent. It might be mentioned at this point that blood samples from the same rabbit taken on subsequent days gave practically identical values. The colorimetric and the manometric methods gave good agreement (Table I).

A qualitative discrepancy seems to exist in respect to the values for oxidized glutathione. Fujita and Numata (15) report in some instances as much as 55 per cent of the total glutathione in a tissue in the oxidized state, while the other authors listed in Table I were unable to detect any appreciable amount of oxidized glutathione.

Among the tissues of the eye we found (Table II) by far the highest concentrations of glutathione in the lens, in agreement with the data for reduced glutathione given by Fujita and Numata (15). Determinations of the separated nuclear (about 66 mg. per cent) and cortical portions

(about 500 mg. per cent) showed a considerably higher concentration in the latter. Similar results were obtained for rabbit lenses by Rosner *et al.* (18). An even greater inequality of distribution was found in the cornea. The level in the isolated epithelium was 110 mg. per cent and in the stroma 5 mg. per cent. The amount found in the whole tissue is as low as 23 mg.

TABLE I\*

*Comparison of Glutathione Contents in Various Organs*

The figures are in mg. per 100 gm. of tissue, fresh weight.

Tissue	Rabbit				Rat							
	Colorimetric	Gly-oxa-lase	Colori-metric		Col-ori-met-ric	Gly-oxa-lase	Colorimetric			Glyoxalase		
			GSH†	GSSG†			GSH	GSSG	GSH			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
Kidney	75, 69, 74, 95, 56, 100, 125, 80, 100	104				100	162.8	6	101.4	134		53
Lung	50, 125	100			140				77.6			
Heart	45, 100	64	78.3	44.5	60	65	52.2	36.8	84.2		67, 64	65, 62
Spleen	77.5				130	125				100		92, 60, 73, 76
Muscle	50, 39, 39, 38.5-50, 42	50, 78	55.3	43.7								
Blood	24, 20, 36, 28, 20, 30, 16- 36, 20, 23, 24, 26, 25, 27		36.8	41.6								
Liver					280		202	43		116- 236	177, 164, 175	174, 187, 177

\* Bibliographic references are as follows: Columns 2, 3, 6, 7, present authors; Columns 4, 5, 8, 9, Fujita and Numata (15); Column 10, Fujita and Numata (16); Column 11, Woodward (6); Column 12, Dohan and Woodward (7); Column 13, Schroeder and Woodward (17).

† GSH = glutathione; GSSG = oxidized glutathione.

per cent, since nine-tenths of the bulk of the cornea is made up by the stroma. The glutathione content in the retina (75 mg. per cent) is comparable to the concentration in the brain and various glands (15). Low values were found in the iris (27 mg. per cent) and in the ciliary body (29 mg. per cent).

In the lens and retina of the unborn calf (7 months of gestation and at term) a glutathione concentration of approximately the same order of

magnitude was found as in the adult animal (Table II). The concentration in the cornea was apparently considerably lower than in adult tissue, since a negative nitroprusside test was obtained in extracts from corneal tissue of sufficient quantity to make it possible to detect as low a concentration as 5 mg. per cent. It should be mentioned, however, that the glutathione content of the lens of the rabbit has been found to increase from 50 to 200 mg. per cent in the first days after birth (Rosner *et al.* (18)).

TABLE II  
*Glutathione Content of Tissues of Bovine Eye*

Tissue	Colorimetric method		Glyoxalase method, GSH
	GSH	GSSG*	
	mg per cent	mg per cent	mg. per cent
Lens. Whole ..	266, 310, 268, 248, 300, 450, 332		212.5, 250, 300, 175, 290, 200
" Cortex..	520, 570, 388, 450, 492, 476	0, 45, 105, 0, 0	
" Nucleus . .	69, 79, 67, 100, 89, 66, 64	54, 71, 44	
" Whole (embryonic)	270, 400		
" " (rabbit).	350		
" Nucleus (rabbit)	120	74	
" Whole	25, 21, 23		25, 29
" Epithelium	130, 78, 84, 136, 150, 140, 104, 112, 110, 127	0	150, 178, 136, 150
" Stroma	7.1, 5, 4, 6, 3	0	5, 7, 4
" Epithelium, em- bryonic	<5		
Retina. . . .	74, 70, 74, 75, 48, 73, 69, 91, 83, 85, 95	0	63, 60, 50, 108, 99
" Embryonic.....	60		
Ciliary body. ...	30, 28, 33, 25, 39, 21, 33, 36, 25, 22, 30, 34, 33	15.5, 29	23, 30, 30, 22
Iris .	20, 28, 33, 30, 25, 30, 22	18	14, 21, 15.5, 20
Aqueous.. . . .	0	0	

\* On reduction of the tissue suspension itself.

Various attempts were made to detect oxidized glutathione, not only in the tissues of the bovine eye, but also in several organs of the rabbit. We followed strictly Fujita and Numata's prescription for the reduction and determination (9) and we also applied Dohan and Woodward's reduction method (7). We tried to vary extractants and the concentrations of their solutions and we tested the organs from animals which were freshly killed and some organs which were removed and stored in the ice box for several hours. Finally, we compared organs from well fed and from fasted animals.

Although in all instances added oxidized glutathione could be quantitatively recovered, no increase of the total glutathione values was observed after reduction of the tissue extracts. Dohan and Woodward's (7) and Ennor's (14) results as well as our own findings make it unlikely that extractable oxidized glutathione is present in the tissues examined, although the discrepancy of Fujita and Numata's results cannot be explained at the present time. These negative experiments did not exclude the possibility that oxidized glutathione is bound to the proteins of the tissues by formation of disulfide bridges between the sulfhydryl groups of glutathione molecules and the SH groups of the proteins. Glutathione bound in this manner should become extractable on reduction of the whole tissue suspension preliminary to extraction. With a reduction technique adapted for this purpose (see previous section) we actually found higher values in the ciliary body, the iris, and the lens nucleus. This finding was checked in the case of the ciliary body with the glyoxalase method and the same increase was obtained, which would indicate that the SH groups which appear after reduction of the tissue suspension actually belong to glutathione and not to cysteine. No increase was observed on reduction of tissue suspensions of the retina or corneal epithelium. Negative results were also obtained with kidney, muscle, and blood of rabbits, the two latter being tissues in which Fujita and Numata found 50 per cent of the total glutathione in the oxidized state (Table II).

*Reduction*—The figures for the glutathione level in tissues represent a steady state rather than a static condition. Therefore, we tried to obtain information about the rates at which glutathione is oxidized or reduced by the tissues of the eye (Table III). First the reduction of oxidized glutathione was examined, both under aerobic and anaerobic conditions. Anaerobically, all tissues reduced added oxidized glutathione, the rate of reduction being high during the 1st hour of incubation and decreasing to practically zero in the following 2 to 3 hours. The total amount of glutathione reduced in this time varied with each tissue. The greatest amount of GSSG<sup>2</sup> was reduced by the corneal epithelium (about  $4 \times 10^{-3}$  milliequivalent per gm. of tissue, fresh weight) and by the lens cortex. The values for retina, ciliary body, and iris were lower, descending in the order mentioned.

Aerobically, we consistently obtained an equally extensive reduction only with suspensions of corneal epithelium. Lens cortex also gave high values, but in one out of three experiments no reduction was observed. The quantity of glutathione reduced by the other tissues aerobically amounted to only a small fraction of that reduced in absence of O<sub>2</sub>, or there was no reduction at all (ciliary body, iris). After reaching the maximum

<sup>2</sup> Oxidized glutathione.



level the amount of reduced glutathione remained constant for several hours under anaerobic conditions; aerobically a gradual decrease was observed on prolonged incubation. This is apparently due to a non-enzymatic oxidation, since the glutathione can be recovered on reduction with  $H_2S$  or by electrolysis and since the rate of disappearance is the same with boiled and with fresh tissue (Table III).

The high reducing capacity of the corneal epithelium was further analyzed. We found that the activity is lost when suspensions of corneal epithelium in Ringer's solution are heated for 20 minutes at  $66^\circ$  and when

TABLE IV

*Reduction of Oxidized Glutathione by Various Tissue Preparations; Reactivation of Inactive Tissue Residues by Heat-Stable Extracts*

Residue and extract corresponding to 500 mg. of tissue, fresh weight, per sample; oxidized glutathione added, 2 mg. per sample; incubation, 30 minutes at  $37.5^\circ$ .

Organ used for preparation		Reduced glutathione recovered after incubation	Average increase
Residue	Extract		
		mg.	mg.
Cornea		0.4,* 0.6, 0.5	
	Cornea	0.4,* 0.4, 0.3, 0.5	
Cornea	"	1.3, 1.5, 2.0	+0.7
	Liver	0.6,* 0.5, 0.6	
Cornea	"	2.5, 1.5, 2.9	+1.5
	Kidney	0.2,* 0.1, 0.2	
Cornea	"	1.3, 1.0, 1.8	+0.7
Retina		0.6*	
"	Cornea	2.5	+1.5
Ciliary body		0.1*	
" "	Cornea	0.7	+0.2

\* The values listed for residue and extracts alone are essentially the same as those obtained before incubation.

they are allowed to stand at room temperature for 3 hours or in the ice box for about 10 hours. After heating, the activity is irreversibly lost but the two latter preparations can be reactivated by addition of tissue extracts. As such, we used the supernatant fluid which is obtained by centrifuging suspensions of corneal epithelium and of various other tissues in Ringer's solution after heating for 20 minutes to  $70^\circ$ . Similar extracts, from fresh, unheated corneal epithelium, themselves frequently showed considerable reducing capacity.

On comparison of the reactivating capacity of extracts from various tissues, high activities were observed when extracts from liver and kidney and from corneal epithelium itself were added to the reversibly inactivated

residue of corneal epithelium. Extracts from retina, ciliary body, and iris and from red blood cells and serum and also solutions of glucose and of glucose + cozymase were devoid of a significant reactivating effect (Table IV). Extracts from corneal epithelium also reactivate residue prepared from kidney or retina. However, only a slight reactivation of the residue from ciliary body could be detected. Concerning the specificity of the reducing system of the corneal epithelium, it should be mentioned that cysteine is reduced at a comparable rate.

#### DISCUSSION

With a glutathione concentration of 400 mg. per cent in the lens cortex and of 5 mg. per cent in the corneal stroma, the values for the GSH content of the tissues of the eye are scattered over the entire range of the known glutathione levels. In two instances in which further comparison was possible, the distribution between the simple components of a single tissue was found to be of remarkable inequality. The concentration in the lens cortex, for example, is about 5 times higher than in the lens nucleus and that in the corneal epithelium, 20 times higher than in the stroma of the cornea. Since the number of cells in the stroma is not less than one-half of that in the epithelium, the ratio for the glutathione content as calculated per cell would still be as high as 10:1. Although the case of the cornea might be an extreme, such results definitely show that the glutathione determinations carried out with extracts from complex tissues are inadequate or even misleading if used as the basis for a functional correlation. Just in the case of the corneal epithelium, however, a functional interpretation should be taken into consideration, not only because the epithelium provides a relatively homogeneous cell material, but also because the high GSH level does not appear before the intrauterine development of the calf is completed. Unfortunately, very little is known about the functions of the corneal epithelium. At the present time one can hardly conjecture whether the particular activity of the cell which ensues simultaneously with the rise of the glutathione level is of a more specific nature, *i.e.* secretory processes, or merely the equivalent of the physiological wear and tear which set in on exposure of the corneal surface.

On reduction of the extracts of the various tissues studied, we were unable to detect an increase in the amount of glutathione beyond the limits of the methodical error. We had to conclude that in these extracts no glutathione is present in the oxidized state, confirming the results of Woodward and of Ennor (14). Other authors claim increases of 10 to 20 per cent of the total glutathione after reduction, but many of these results have been criticized by Woodward (6) for technical reasons. However, Fujita and Numata's figures for the total glutathione content are in some instances

as much as 100 per cent higher than the values for reduced glutathione alone (rabbit muscle and blood). It should be emphasized that the use of  $H_2S$  which was Woodward's main objection to the Fujita and Numata reduction method does not interfere with the colorimetric determination (see previous section). We failed to find any explanation for the disagreement of Fujita and Numata's figures, although we attempted to eliminate discrepancies due to technique by performing the tests under a great variety of experimental conditions. The only procedure which gave rise to an increased content of reduced glutathione in some of the tissues was the reduction with  $H_2S$  of suspensions of the tissue itself. This finding could be explained by assuming that in some tissues a small fraction of the glutathione is bound to inextractable cell constituents by a linkage which is removed on reduction, most likely a disulfide link between GSH and SH groups of some proteins. The fact that this bound form of oxidized glutathione occurs in a few tissues only suggests rather specific conditions for its formation.

The presence of glutathione in the tissues of the eye almost entirely in its reduced state would seem to be plausible in view of the high rate at which oxidized glutathione is reduced in preparations of the tissues of the eye, which was also observed with liver and kidney by Hopkins and Elliott (19).

Several attempts have been made by other authors to identify the components of the system which reduces oxidized glutathione. Mann (20) concluded that GSSG is reduced by glucose dehydrogenase in ground liver. Meldrum (21) found that intact erythrocytes reduce glutathione not only with glucose, but also with mannose and fructose. Meldrum and Tarr (22) showed that glutathione can act as hydrogen acceptor for the oxidation of hexose monophosphate through the Warburg-Christian system. Morgulis (23) attributes the maintenance in the reduced state of the glutathione in the blood to the fluoride-sensitive part of glycolysis.

We attempted a further analysis only in the case of the corneal epithelium, which contains the most active reducing system among the tissues of the eye. It was found to consist of a heat-labile and a heat-stable fraction, the first containing the enzyme, the second the substrate and possibly other heat-stable components. The heat-stable fraction of the corneal epithelium can be substituted for by analogous extracts from some other tissues. A very potent heat-stable extract is obtained from liver. According to Mann (20), this extract should contain the heat-stable fraction for the reduction of glutathione by glucose dehydrogenase of the liver. The fact that reduction takes place with the enzyme of the corneal epithelium would suggest that the same enzyme is also present in the cornea. However, on addition of glucose alone and with diphosphopyridine nucleo-



tide to the corneal residue no reduction occurs. This does not necessarily exclude the presence of glucose dehydrogenase, since this system apparently requires another coenzyme. However, a reduction by a glycolytic mechanism, as suggested by Morgulis (23), appears improbable from this experiment. Meldrum (21) also excluded glycolysis as a reducing mechanism since he failed to observe an inhibition of the reduction by fluoride or a reactivation by various glycolytic intermediates.

#### SUMMARY

1. The concentrations of reduced glutathione found in the tissues of the eye, expressed as mg. per 100 gm. of tissue, fresh weight, are as follows: lens cortex 388 to 570, lens nucleus 64 to 100, corneal epithelium 78 to 178, corneal stroma 3 to 7.1, corneal epithelium from fetal calf <5, retina 50 to 108, ciliary body 21 to 39, iris 14 to 33, aqueous 0.

2. No oxidized glutathione was detected in tissue extracts. However, the amounts of reduced glutathione found in the lens nucleus, the ciliary body, and the iris increased by 50 to 100 per cent after reduction of the tissue suspension itself with  $H_2S$ .

3. The reduction of oxidized glutathione by the tissues of the bovine eye has been investigated. A high reducing capacity was found in the corneal epithelium and in the lens cortex, whereas retina, ciliary body, and iris were less active.

4. The reducing system of the corneal epithelium was found to consist of a heat-stable and a heat-labile component. The effects of these components can be induced with preparations from other tissues. Glucose and cozymase were not found capable of replacing the heat-stable factor.

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ably high oxidation rate with hydroquinone alone. The oxygen uptake of preparations from both central and peripheral portions of the lens in the presence of cytochrome *c* and hydroquinone was inhibited by 0.005 M cyanide. The quantitative extent of this inhibition proved to be highly irregular and would require further investigation for its elucidation.

TABLE I

*Oxygen Uptake at 38° of Preparations of Beef Lens Cortex*

Per flask, 1 ml. of enzyme solution; hydroquinone, 0.5 ml. of a 2 per cent solution; cytochrome *c*, 0.5 ml. of a 1 per cent solution.

	Oxygen uptake, c.mm.					
	Preparation A		Preparation B		Preparation C	
	30 min.	60 min.	30 min.	60 min.	30 min.	60 min.
Enzyme.....	3	2	7	9	1	3
" + hydroquinone.....	2	4	8	11	2	3
" + cytochrome <i>c</i> .....	3	3	8	10	2	3
" + hydroquinone + cytochrome <i>c</i> .....	55	96	50	90	39	83

From our experiments, especially those with preparations from the lens cortex, the presence of cytochrome oxidase activity seems to be strongly indicated. Since the paper of von Euler *et al.* is devoid of data on experimental technique regarding the estimation of cytochrome oxidase activity, the disagreement cannot now be explained.

## SUMMARY

The oxidation of hydroquinone by preparations of the bovine lens is greatly accelerated upon addition of cytochrome *c*. This suggests the presence of cytochrome oxidase in this tissue.

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# THE PURIFICATION OF ANGIOTONIN

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The importance of angiotonin, a pressor substance resulting from the interaction of the  $\alpha_2$ -globulin fraction of serum (1, 2) and renin, in its relationship to arterial hypertension warrants further efforts to elucidate its chemical constitution. Systematic investigations on the formation and inactivation of angiotonin carried out in this laboratory by enzymatic methods during the last 3 years (3-5) revealed some interesting information regarding certain groupings in the angiotonin molecule, but such methods should be supplemented by more direct chemical approaches. Until the compound can be prepared pure and until its empirical formula is known, chemical analytical methods to determine its concentration in blood and other body fluids will necessarily be unreliable. Further, attempts to reconstruct the molecule from degradation products will be unsuccessful, since these degradation products or certain reactions, so far attributed to angiotonin, might be due to the presence of impurities. Although purity has not been achieved, in the following there are described a number of procedures by means of which we have succeeded in concentrating angiotonin beyond the stages so far reported.

## EXPERIMENTAL

A few general remarks on the characteristics and technique in working with angiotonin preparations will first be given. The pressor principle seems to be a polypeptide (3) and is very susceptible to bacterial action. Aqueous solutions of angiotonin, if not sterilized by heat or filtration through a Seitz filter, are not infrequently inactivated within a few hours at room temperature. When kept in the frozen state, even if contaminated, the material will maintain its activity for several weeks.

Although angiotonin appears to be heat-stable (1), *i.e.* it is not inactivated by boiling its aqueous solutions, it is nevertheless desirable to concentrate such solutions at temperatures not exceeding 20°. The use of the conventional capillary tube to prevent bumping during concentration under reduced pressure is contraindicated, since it causes serious losses in activity, even if the operation is carried out under anaerobic conditions. We are not prepared to explain this phenomenon. Long applicator sticks and capryl alcohol in liberal amounts are a suitable substitute.

Previous reports from this laboratory (1) and elsewhere (6) state that

angiotonin is unstable in alkaline media and fairly stable in acid solution. It is therefore recommended that the working range be kept within 7 pH units; namely, pH 1.5 to 8.5. Degrees of alkalinity higher than pH 8.5 result in serious loss.

The following describes a representative experiment on the preparation and purification of angiotonin. The preparation of renin-substrate is briefly described. Renin was prepared by a method previously given (7).

*Renin-Substrate*—70 liters of fresh hog blood were collected in eight buckets and allowed to clot. The clots were suspended in cheese-cloth bags and allowed to drain in a cold room overnight. The bags were discarded and the serum and red cells pooled in one or two buckets. After standing for 24 hours, the serum was decanted from the red cells and once more allowed to stand in the cold room overnight. After decanting the serum from the small amount of red cells, it was brought to pH 6.0 by addition of 20 cc. of N sulfuric acid per liter of serum. The volume of serum usually amounted to 20 to 25 liters. With efficient but not too rapid mechanical stirring, an equal volume of 3 M  $(\text{NH}_4)_2\text{SO}_4$  was then added through a large separatory funnel in such a way that the tip of the funnel extended below the surface of the liquid and the rate of addition did not exceed 2 liters per hour. The ammonium sulfate should not be added faster than this, because subsequent filtration becomes greatly prolonged.

Now, 200 gm. of Hyflo Super-Cel were added and the mixture filtered on ten large (18 cm.) Büchner funnels with coarse filter paper. The precipitate consisting mostly of Hyflo Super-Cel and  $\gamma$ -globulin was discarded, and the filtrate brought to 2.0 M by the addition of the calculated amount of solid ammonium sulfate. (The volume of 1.5 M filtrate in liters multiplied by 66 gives the amount of  $(\text{NH}_4)_2\text{SO}_4$  in gm. to be added to bring the solution to 2 M.) The salt was finely powdered to facilitate its slow addition. The mixture was filtered on large Büchner funnels with No. 54 Whatman filter paper with a thin layer of Hyflo Super-Cel. The precipitate was pressed dry, removed from the funnels, and dissolved in 2000 cc. of water. When all of the protein had dissolved, the mixture was filtered through coarse paper. The filtrate was a clear solution with blue fluorescence. It was placed in cellophane bags and dialyzed against running tap water until free from ammonium ion. After dialysis for 12 to 24 hours tests with Nessler's reagent were usually negative.

The protein content of the dialyzed solution was determined by the biuret method of Kingsley (8) or the micro-Kjeldahl method and the solution was diluted to contain 2 per cent total protein. This was the renin-substrate solution generally employed for the preparation of angiotonin.

*Incubation of Renin-Substrate with Renin*—A renin solution in normal

saline was prepared according to the procedure previously described (4, 5). It was found best to keep the renin in the dry (lyophilized) state until needed. A 1 per cent solution in saline was then prepared. If the renin preparation was of unknown potency, it was assayed for its renin and angiotonase content. Generally, it was found to be 1000 to 1200 renin units per mg. of N.

The renin-substrate solution was brought to 30° in an enamel pot and 4.0 cc. of the renin solution added with stirring to each liter of substrate. The reaction was allowed to proceed for 10 to 15 minutes, when the pH of the solution was adjusted to 5.5 (glass electrode) with the necessary amount of 2N H<sub>2</sub>SO<sub>4</sub>. It was then poured into a number of smaller pots containing not more than 1000 cc. each and placed in a preheated autoclave. As quickly as possible, the sterilizer was closed and the temperature raised rapidly. The denaturation of the proteins was found to be complete after 15 to 20 minutes at 20 pounds steam pressure. The pots were taken out, allowed to cool, and the mixture filtered through coarse paper with suction. The clear colorless filtrate contained about 1 pressor unit per cc.<sup>1</sup> Roughly 10 liters of this solution were obtained from 20 liters of serum.

*Angiotonin I*—When not immediately further purified, this dilute angiotonin solution was again sterilized in 3 liter Erlenmeyer flasks in the autoclave (15 minutes, 20 pounds) and stored in the cold. Two or three batches were combined and concentrated under reduced pressure at 15° to one-tenth their original volume. When this solution was heated in a sterilizer, an additional amount of denatured proteins formed, which was removed by filtration through coarse paper with Filter-Cel. The filtrate was assayed (4) and diluted to contain 10 pressor units per cc. On subsequent heat sterilization, such solutions remained clear, though they were generally not colorless.

*Angiotonin II (Silver Precipitation)*—A number of preliminary experiments on the precipitation of angiotonin were carried out on angiotonin I. The important analytical data on several successive silver precipitations are reproduced in Table I from which it can be seen that the precipitation repeated more than twice results in a large loss of activity. The following procedure for the purification of angiotonin was found to give consistently good results.

1000 cc. of the angiotonin solution containing approximately 10,000 pressor units were adjusted to pH 7.0 and 100 cc. of saturated basic lead acetate were added. A bulky, white precipitate formed, which was centrifuged and washed with 500 cc. of water. The precipitate was set aside

<sup>1</sup> 1 pressor unit is defined as the quantity necessary to raise the arterial pressure of a pithed cat 30 to 50 mm. of Hg; all pressor units reported in this paper are relative to a reference standard.

and the supernatant and washings combined. This clear solution (Filtrate A) was brought to pH 1.5 with 8 N sulfuric acid and a slight excess of silver nitrate (50 per cent aqueous solution) added. The mixture was allowed to stand at room temperature for 1 hour, the clear supernatant decanted, and the residue centrifuged. The supernatants were combined and filtered through coarse paper with Filter-Cel. The clear filtrate was then brought to pH 7.8 to 8.0 with saturated barium hydroxide, and the precipitated silver salt centrifuged and washed three times with water. The washed precipitate was suspended in about 300 cc. of water, the pH was then brought to 4.0 with 5 N sulfuric acid, a few drops of capryl alcohol added, and the mixture cooled in ice. Hydrogen sulfide was blown through the suspension for 15 minutes, a small amount of Filter-Cel added, and the precipitate consisting of silver sulfide and barium sulfate removed by filtration through coarse filter paper with suction. The filtrate was concen-

TABLE I

*Preliminary Experiments on Precipitation of Angiotonin with Silver*

A crude angiotonin sample was precipitated three times with silver nitrate by the procedure outlined in the text. After each precipitation small samples were withdrawn for analytical purposes and the appropriate corrections incorporated in the values for total nitrogen and total pressor units.

	Total nitrogen	Total pressor units	Pressor units per mg. N
	mg.		
Original material.....	432	4220	9.8
1st Ag pptn.....	118	3000	25.4
2nd " " .....	72	1950	27.1
3rd " " .....	22	685	31.0

trated under reduced pressure to about 50 cc., and the excess sulfuric acid removed with barium hydroxide by adjusting the pH to 4.0, Congo red paper being used as indicator. The precipitate was removed by filtration through a Seitz pad (Filtrate B). The clear colorless filtrate was frozen and dried in the frozen state. The residual light hygroscopic powder (angiotonin II) could be kept for months at room temperature in a dry atmosphere (desiccator or sealed bottle).

*Recovery from Lead Precipitate*—The bulky, white, lead precipitate was suspended in 200 cc. of water, 15 gm. of solid ammonium sulfate added, and the mixture stirred until a homogeneous suspension was obtained. After standing in the ice box for several hours, the precipitated lead salts were removed by centrifugation and the clear supernatant treated as described above for Filtrate A.

The analytical data for angiotonin II are recorded in Table II. These

values are average figures determined on twenty batches. A solution of angiotonin II gives a positive, though not very strong, Sakaguchi reaction and an intense coloration with diazotized sulfanilic acid. Color reactions for all other known amino acids were negative.

*Angiotonin III*—Precipitation with mercuric chloride by the procedure usually employed for the precipitation of histidine (9) did not effect any concentration of the pressor principle. 70 to 90 per cent of the angiotonin was, however, recovered. Analytical data on samples so treated are given in Table II (angiotonin III).

*Angiotonin IV*—100 mg. of angiotonin III (mercury precipitate) were dissolved in 2.0 cc. of water and absolute methyl alcohol added drop by drop until the solution became cloudy. A few drops of water were then added to form a clear solution which was freed from a small amount of insoluble material by filtration. 100 mg. of recrystallized nitranilic acid were then added in small amounts, resulting in the immediate precipitation

TABLE II  
*Analytical Data Obtained during Various Stages of Purification Procedure*

	Angio- tonin I	Angio- tonin II	Angio- tonin III	Angio- tonin IV	Angio- tonin V	Angio- tonin VI
Total N, mg. per cc.....	0.778	0.758	0.502	0.404		0.125
Amino " " " ".....	0.173	0.127	0.092	0.068	0.171	0.048
" acid carboxyl, mg. per cc....	0.057	0.084	0.070	0.068	0.055	0.049
Pressor units per cc.....	10.0	33.3	23.8	21.0	15.0	10.0
" " " mg. NH <sub>2</sub> -N.....	58.0	262.0	260.0	306.0	880.0	210.0
NH <sub>2</sub> -N:COOH-N.....	3.04	1.35	1.20	1.0	3.1	0.98

of an apparently crystalline material. The solution was allowed to stand in the ice box overnight, when it was centrifuged and washed with absolute methanol until the supernatant remained practically colorless. After being washed with absolute ether, the yellow precipitate was dried in the open air. It was suspended in 250 cc. of water and dissolved by warming with occasional shaking. Sufficient acid-washed pure lamb's wool was added to extend beyond the level of the liquid and the mixture shaken on the shaking machine for 3 to 6 hours (10). The clear solution was decanted and concentrated under reduced pressure to approximately one-tenth its original volume. During concentration the solution became slightly yellow owing to traces of nitranilic acid which apparently could not be removed with wool even if a large excess was used. Attempts to remove the residual coloration by adsorption on aluminum oxide failed. Since the amount of nitranilic acid present was negligible, the solution was frozen and dried in the frozen state. 79 per cent of the pressor activity was recovered (Table II).



*Angiotonin V*—A suitable quantity of angiotonin IV was dissolved in a sufficient amount of water to give a solution containing 20 to 30 pressor units per cc. Silver and mercury precipitations were carried out as described above. The final product still contained traces of nitranilic acid but its pressor activity per mg. of  $\text{NH}_2\text{-N}$  had more than doubled (Table II).

*Angiotonin VI*—25 cc. of a solution of angiotonin II containing 595 pressor units were diluted to 110 cc. with absolute methyl alcohol. It was then allowed to flow through a column of aluminum oxide (according to Brockmann)  $20 \times 110$  mm., which had previously been washed with 80 per cent methyl alcohol. The column was washed with three 150 cc. portions of warm water and the filtrate collected separately. The filtrates were concentrated to approximately one-third their original volume and the activity was found to be associated with the first and second fractions only. 50 per cent of the activity could be recovered (Table II).

TABLE III

*Course of Acetylation of Angiotonin with Ketene*

The experiment was carried out according to the procedure outlined in the text. The column headed "Reaction time" designates the length of time during which ketene was passed through the reaction mixture. A control retained 10 pressor units per mg. of N.

Reaction time	Total N	Amino N	Physiological activity
<i>hrs.</i>	<i>mg. per cc.</i>	<i>per cent of total N</i>	<i>units per mg. N</i>
0	0.755	19.7	10
1	0.600	15.0	None
5	0.510	14.1	"

*Acetylation of Angiotonin with Ketene*—A solution of angiotonin II containing 10 pressor units per cc. was cooled in ice. The pH was adjusted to 4.5 and maintained at this pH throughout the experiment by means of sodium acetate-acetic acid buffer. Ketene prepared according to the procedure of Williams and Hurd (11) was passed through the solution at a slow but constant rate. Samples for the determination of total nitrogen, amino nitrogen, and physiological activity were withdrawn at frequent intervals (Table III). The acetylated product as well as that derived from mild saponification with 0.01 N NaOH was physiologically inactive. We attribute our inability to recover a physiologically active material to the action of the alkaline medium used for saponification.

## DISCUSSION

The procedure outlined in the experimental portion led to the concentration of the pressor principle with the elimination of a number of undesirable

contaminants. Because of its great solubility in water and polar solvents, the pressor principle was difficult to obtain free of inorganic salts. This could only be accomplished by precipitation with specific reagents, leaving the impurities in the filtrate.

Evidence presented elsewhere (3) points to the polypeptide nature of angiotonin; hence one may expect the criteria of purity to be limited to elementary analysis, phase rule relations, and possibly elementary analyses of crystalline derivatives. The problem therefore arises which element, functional group, or specific chemical reaction when related to physiological activity could serve as a guide for purification.

Of the possible elements present in angiotonin only carbon, hydrogen, and nitrogen could be identified. Cruz-Coke and Restat (12) have reported the presence of sulfhydryl groups in angiotonin samples, a finding which could not be substantiated in this laboratory. It is possible that their observations may be attributed to the use of crude angiotonin samples containing varying amounts of soluble denatured proteins or large amounts of free amino acids. Qualitative reactions for sulfhydryl on silver-precipitated angiotonin (angiotonin II) were entirely negative.

Since angiotonin contains nitrogen, the pressor activity per mg. of nitrogen may serve as an index for purification. This nitrogen is to be attributed to peptide nitrogen,  $\alpha$ -aminocarboxyl nitrogen, and primary amino nitrogen, since all attempts to demonstrate other nitrogenous materials such as pyrimidines, purines, pyrrole derivatives, quaternary ammonium salts, etc., have failed. Its very mode of formation (it is the product of catheptic action on blood globulins) suggests that a number of similar substances, probably polypeptides, are formed simultaneously. The three types of nitrogen-containing groups, peptide nitrogen,  $\alpha$ -aminocarboxyl nitrogen, and primary amino nitrogen, are therefore a measure of all polypeptides and amino acids in this mixture.

The association and correlation of angiotonin pressor activity with peptide linkages have been demonstrated by the inactivation of angiotonin by proteolytic enzymes (3). The rate of increase in amino nitrogen during enzymatic digestion (Fig. 1) is widely different from the rate of inactivation; hence it may be concluded that the sensitive peptide bonds in the active principle do not seem to be detectable by common analytical procedures. The amino nitrogen on complete acid hydrolysis amounts to 3 or 4 times that of the unhydrolyzed material.

These observations may be explained in two ways. Angiotonin may be assumed to be a polypeptide of high molecular weight (2000 or higher), in which case the enzymatic hydrolysis of one sensitive peptide bond would be beyond the experimental error of the amino nitrogen determination yet sufficient to inactivate the material completely. Against this concept

it should be pointed out that angiotonin readily diffuses through cellophane membranes, which is indicative of a lower molecular weight. In this connection it should be noted that the action of crystalline pepsin on proteins results in the formation of peptide fragments with a molecular weight of approximately 1000 as demonstrated by Tiselius and Eriksson-Quensel (13). Although pepsitensin and angiotonin are not identical (4, 14), the striking similarity in their chemical and pharmacological behavior would warrant the assumption that they are peptides of similar molecular weight.

On the other hand, angiotonin samples may be regarded as a mixture of, for example, tetra-, penta-, and hexapeptides with angiotonin constituting only a small portion of the mixture. This is in agreement with the fairly

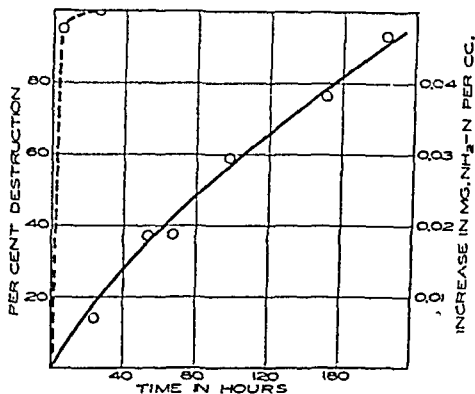


Fig. 1. Increase in amino nitrogen (solid line) during peptic digestion compared to decrease in physiological activity as per cent of total (dotted line). The enzyme concentration was 0.266 mg. of (crystalline) pepsin N per cc. of test solution.

consistent values of amino nitrogen (1.7 to 2.0 per cent) for the purest angiotonin preparations. On the assumption of one free amino group per molecule the average molecular weight for this mixture can be estimated as between 500 and 1000.

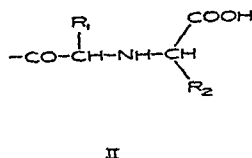
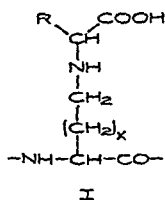
The evidence for the existence of a free amino group in the angiotonin molecule rests on the observations of Croxatto and Croxatto (15) who demonstrated that it is rapidly inactivated by purified yeast aminopeptidase. They drew the conclusion that, since their aminopeptidase was free of endopeptidase activity and since this enzyme requires a free primary amino group in its substrates, angiotonin must contain a free terminal amino group. In support of this finding we have demonstrated that chymotrypsin can inactivate angiotonin (3) and that the most probable

explanation for this observation is to be sought in the exopeptidase activity of this enzyme. Although these experiments seem quite convincing to us, evidence based on enzyme studies should be confirmed by more direct chemical methods.

Benzoylation of angiotonin by the Schotten-Baumann reaction and its associated inactivation as reported by Braun-Menendez and coworkers (6) cannot be accepted as evidence for a free amino group, since it is well known that this reaction necessitates a strongly alkaline medium, so insuring almost instantaneous inactivation. The experiments of Braun-Menendez *et al.* would only be of value if the authors had been able to regenerate the pressor substance from the benzoylated material, which for the reasons given could not be accomplished.

The inactivation of angiotonin by ketene under exceedingly mild conditions demonstrates the presence of atomic groupings such as phenolic hydroxyl and free primary amino groups. Since we were unable to demonstrate the presence of tyrosine or dihydroxyphenylalanine in completely hydrolyzed angiotonin samples, the inactivation of angiotonin samples by ketene represents conclusive proof for the presence of a free amino group in the pressor principle.

Consideration of methods of purification of angiotonin suggests that it is associated with what is known as the "histidine fraction." Since the major portion of amino acids precipitating in this fraction is histidine and perhaps a small amount of arginine, dicarboxylic acids, and phenylalanine, the amino acid carboxyl must be attributed to angiotonin or the amino acid impurities. That angiotonin or an associated polypeptide may give rise to carbon dioxide by the ninhydrin method appears to be remote (16), but is nevertheless possible if angiotonin or the polypeptide impurity contains an arrangement represented by Formula I or II. If such a structure as



well as the amino nitrogen is associated with pressor activity, the ratio of amino nitrogen to amino acid carboxyl must approach a whole number or a fraction thereof as the activity per mg. of amino nitrogen increases during the purification procedure. If the amino nitrogen, but not the amino acid carboxyl, is associated with physiological activity, this ratio will increase

rapidly as the activity per mg. of amino nitrogen increases. A number of analytical data were taken at random and the above defined ratio plotted against the activity per mg. of amino nitrogen. The data are reproduced in Fig. 2, from which it can be seen that the ratio  $\text{NH}_2\text{-N}:\text{COOH-N}$  increases to a high value. The original ratio of 3.0 falls to about 1.0 with a slight increase in physiological activity and then increases slowly. The sudden initial drop in the numerical value of this ratio is due to the silver and mercury precipitation which will tend to concentrate free histidine in this fraction. Further purification beyond this stage results in the partial elimination of histidine, particularly if the precipitation is carried out at a

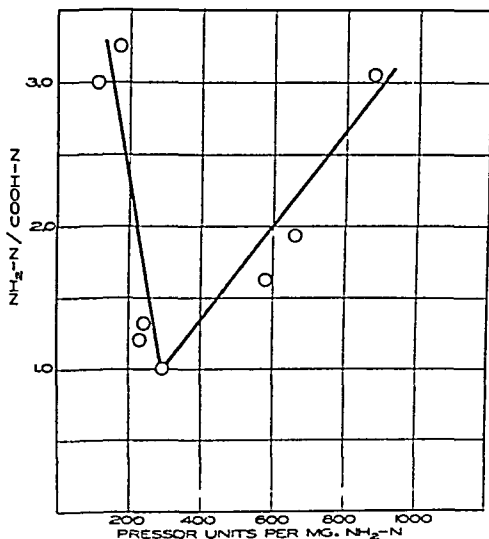


FIG. 2. Pressor units per mg. of amino nitrogen for arbitrary stages in the purification procedure plotted against the ratio of amino nitrogen to amino acid carboxyl.

slightly higher pH and with elimination of salts precipitating below pH 5.0, with a consequent rise in the ratio of amino nitrogen to amino acid carboxyl. The data at hand do not permit a definite statement regarding the possibility of a further increase in the value of this ratio which may still approach a whole number as the activity per mg. of amino nitrogen increases. The indications are, however, that the amino acid carboxyl is not associated with the angiotonin molecule.

The authors are indebted to Miss Doris Brown, Mr. Robert M. Sanders, and Mr. Donald Grove for their valued assistance, and to Mr. and Mrs.

C. L. Bradley of Cleveland for defraying part of the expense of this investigation.

#### SUMMARY

The preparation of renin-substrate by a simplified procedure is described. A detailed description for the preparation of crude angiotonin is given. The purification of angiotonin was effected by precipitation as silver salt in acid and neutral solution, followed by regeneration and removal of the silver with hydrogen sulfide. It was shown that angiotonin can also be precipitated with mercuric chloride in neutral solution, suggesting that it is associated with the "histidine fraction."

Attempts to demonstrate basic amino acids other than histidine in hydrolyzed and unhydrolyzed angiotonin samples failed. Purified preparations were found to be devoid of tyrosine, phenylalanine, and dihydroxyphenylalanine (dopa).

Further evidence was presented for the presence of at least one free amino group associated with pressor activity. Angiotonin was found to lose its pressor action when acetylated with ketene. The activity could not be restored by alkaline saponification of the acetylated material.

Analytical data on the various fractions obtained during the course of purification were interpreted as suggesting that the partially purified angiotonin is a mixture of tetra-, penta-, and hexapeptides and that the active principle constitutes only a small part of them.

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# STUDIES ON THE SEX HORMONES

## I. THE QUANTITATIVE ESTIMATION OF ESTRONE IN PURE SOLUTION

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(Received for publication, November 27, 1944)

During our work on the sex hormones we have had occasion to prepare the 2,4,-dinitrophenylhydrazone of estrone. The high tinctorial power of this compound suggested that it might be used for the quantitative estimation of estrone. Alcoholic solutions of this 2,4,-dinitrophenylhydrazone exhibit maximum absorption in the ultraviolet region of the spectrum. In 0.1 N alcoholic potassium hydroxide, this hydrazone exhibits maximum absorption at 440 m $\mu$ , a value which lies well within the range of most photoelectrometers and regional spectrophotometers.

### EXPERIMENTAL

*Preparation of 2,4,-Dinitrophenylhydrazone of Estrone*—30 mg. of pure crystalline estrone<sup>1</sup> (weighed on a micro balance) were dissolved in 50 ml. of aldehyde-free ethyl alcohol. To this solution were added 10 ml. of a saturated alcoholic solution of 2,4,-dinitrophenylhydrazine, prepared by refluxing 1 gm. of the hydrazine with 100 ml. of aldehyde-free ethyl alcohol. The mixture was refluxed for 2 hours, at the end of which time 1 ml. of concentrated hydrochloric acid was added and the refluxing continued for an additional 2 minutes. Distilled water was added to the point of incipient crystallization and the solution allowed to cool to room temperature, after which it was chilled to 0° in an ice bath. The precipitated hydrazone was filtered off in a previously weighed and tared micro filter beaker and the precipitate washed with 50 ml. of distilled water containing 1 ml. of concentrated hydrochloric acid. The filter and contents were then carefully dried in a vacuum desiccator over concentrated sulfuric acid and finally weighed. Yield 49.92 mg., or 99.8 per cent of theory.

M.p. 278–280° (uncorrected) with decomposition.<sup>2</sup> Nitrogen, by micro-Dumas method, found 12.4, theory 12.43.

*Spectroscopic Analysis*—4.634 mg. of the above hydrazone, equivalent to 2.78 mg. of estrone, were dissolved in alcohol and this solution made up

<sup>1</sup> We are indebted to Dr. Oliver Kamm of Parke, Davis and Company, who supplied the crystalline estrone used in this investigation.

<sup>2</sup> L. F. King and W. R. Franks report 278–280° (uncorrected) with decomposition (*J. Am. Chem. Soc.*, 63, 2042 (1941)).



to a volume of 100 ml. 1 ml. of this solution, equivalent to 27.8  $\gamma$  of estrone, was diluted to 10 ml. in 0.1 N alcoholic potassium hydroxide and submitted to spectroscopic analysis in a Coleman regional spectrophotometer, model 10-S. Measurements were made in a round cell approximately 19 mm. in diameter, with distilled water as a blank and a 30  $m\mu$  slit. The hydrazone exhibited maximum absorption at 440  $m\mu$  (Fig. 1). The color developed by this compound in alkali is stable for 15 minutes. Longer contact with alkali results in measurable decomposition.

*Preparation of Transmittance-Concentration Curve*—A series of solutions containing increasing amounts of the 2,4-dinitrophenylhydrazone of estrone in 10 ml. of 0.1 N alcoholic potassium hydroxide was prepared and

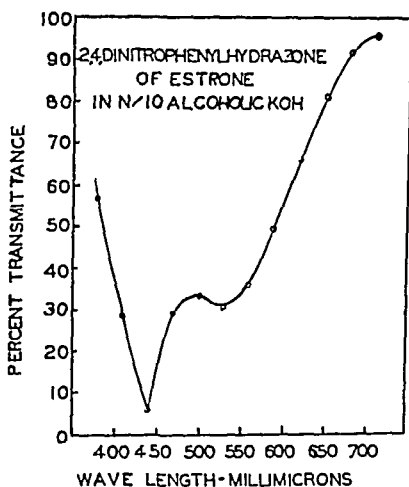


FIG. 1. Transmittance of 2,4-dinitrophenylhydrazone of estrone in 0.1 N alcoholic KOH.

the transmittance at 440  $m\mu$  determined. From these data a transmittance-concentration curve was prepared (see Fig. 2) by plotting transmittance at 440  $m\mu$  against concentration of estrone.

Repeated experimentation has shown that with pure solutions of hydrazone these results may be duplicated within the sensitivity of our particular instrument, i.e., 0.2 per cent transmittance or 0.06  $\gamma$  of estrone.

*Chromatographic Separation of Unchanged Hydrazone from Hydrazone*—Since the quantity of estrone usually present in biological material is small (1.0 to 25  $\gamma$ ), precipitation and filtration methods for the isolation of its 2,4-dinitrophenylhydrazone are impractical. Chromatographic methods seemed to offer the most practical solution to this problem, and were found to permit quantitative separation of hydrazone from hydrazone.

The chromatographic columns used in this laboratory are constructed according to the drawing in Fig. 3. We have found that forcing the solu-

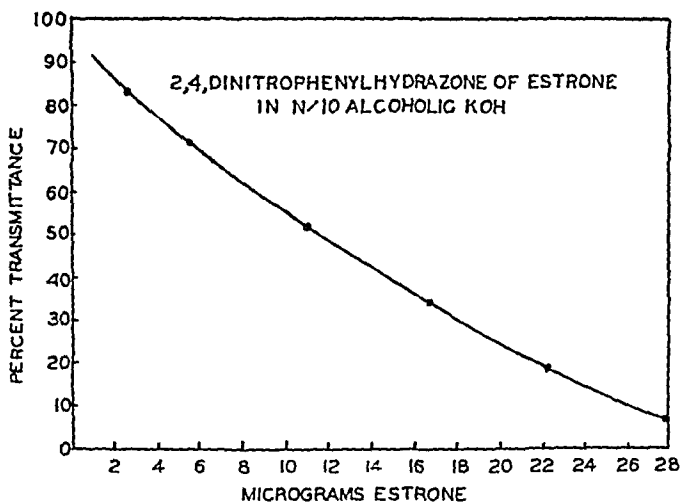


FIG. 2. Transmittance-concentration relationship of the 2,4-dinitrophenylhydrazone of estrone in 0.1 N alcoholic KOH.

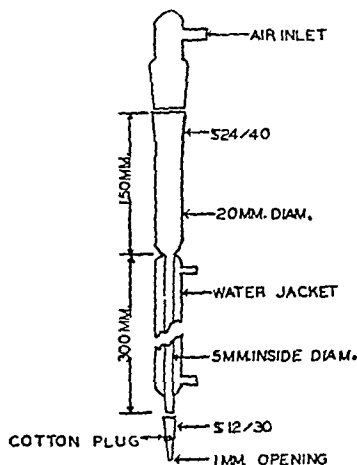


FIG. 3. Water-jacketed chromatographic column

tion to be examined through the adsorbent with air pressure of approximately 500 mm. of mercury is more desirable than the use of suction or

gravity percolation. The adsorbent is most conveniently introduced into the column in the form of a slurry in anhydrous petroleum ether. Application of air pressure then results in a column of uniform density free from air bubbles and channeling. It is important to maintain 2 to 3 mm. of liquid above the adsorbent in the column at all times, since allowing the column to become dry results in channeling of the adsorbent, thereby reducing its efficiency.

The adsorbents used in this work were florasil<sup>3</sup> 100 to 200 mesh, and Merck's aluminum oxide<sup>4</sup> prepared according to Brockmann.

With small amounts of estrone, *i.e.* 1 to 25  $\gamma$ , 1 ml. of an alcoholic solution of 2,4-dinitrophenylhydrazine containing 1 mg. of hydrazine in 1 ml. was added to the estrone in 5 ml. of aldehyde-free ethyl alcohol and the mixture refluxed for 15 minutes. At the end of this period 0.15 ml. of concentrated hydrochloric acid was added and the alcohol removed under reduced pressure, care being taken to prevent loss and insure the removal

TABLE I  
*Recovery of Estrone from Solution*

Estrone used	Per cent transmittance at 440 m $\mu$	Estrone recovered	Per cent recovery
$\gamma$		$\gamma$	
7.21	66.1	7.15	99.16
14.42	41.8	14.42	100.00
21.63	20.7	21.63	100.00
25.96	9.0	25.85	99.57

of all of the alcohol. The residue was dissolved in 10 ml. of benzene with the aid of heat. When the benzene solution had cooled to room temperature, it was transferred to a chromatographic column containing florasil. This column, which had a diameter of 5 mm., had been previously filled with the adsorbent to a height of 200 mm. The reaction flask was washed out with several additional 2 ml. portions of benzene and the washings added to the solution on the column. When all but about 1 mm. of this solution had been forced through the column, an additional 5 ml. of benzene was added and forced through. The column was then washed with 25 ml. of 4 per cent acetone in petroleum ether (b.p. 30–60°). This treatment removed some unidentified material, but did not remove the hydrazone of estrone. The hydrazone of estrone was removed by washing the column with 35 ml. of 10 per cent acetone in petroleum ether. Some unidentified impurities accompanied the hydrazone in this eluate. This

<sup>3</sup> Manufactured by the Floridin Company.

<sup>4</sup> E. Merck, Darmstadt.

eluate was collected separately, and after evaporation of the solvent the residue was dissolved in benzene and passed through a column of aluminum oxide  $5 \times 100$  mm. The column was washed successively with 25 ml. of 1 per cent acetone in petroleum ether and 10 ml. of chloroform. The hydrazone of estrone was then eluted by washing the column with 75 ml. of 1 per cent ethyl alcohol in petroleum ether. After evaporation of the solvent from this eluate, the residue was dissolved in 10 ml. of 0.1 N alcoholic potassium hydroxide and the transmittance of this solution at 440  $m\mu$  determined. The 2,4-dinitrophenylhydrazone of estrone separated from hydrazine in this manner showed no depression of the melting point when melted mixed with an authentic sample of estrone hydrazone, and in addition showed the same type of absorption curve as the pure hydrazone of estrone.

The estrone concentration of four solutions of pure estrone was determined by preparing the 2,4-dinitrophenylhydrazone, separating the hydrazone from unchanged hydrazine in the manner described above, and determining the transmittance of the purified hydrazone in 0.1 N alcoholic potassium hydroxide. The results of these experiments are summarized in Table I. All solvents used in this work were carefully purified and dried unless otherwise stated. The ethyl alcohol was freed of aldehydes by treatment with silver oxide and subsequent distillation from sodium hydroxide pellets. No attempt was made to dry the alcohol, it being approximately 98 per cent after this treatment.

#### SUMMARY

1. The 2,4-dinitrophenylhydrazone of estrone has been prepared and characterized.
2. The concentration-transmittance relationship of this hydrazone in 0.1 N alcoholic potassium hydroxide at 440  $m\mu$  has been determined.
3. A chromatographic method for the quantitative separation of small amounts of the 2,4-dinitrophenylhydrazone of estrone from unchanged hydrazine has been described.
4. A sensitive method for the quantitative estimation of small amounts of estrone in pure solution has been outlined.



# THE METABOLISM OF PHENOLIC COMPOUNDS BY NORMAL AND SCORBUTIC GUINEA PIG LIVER SLICES IN VITRO

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(Received for publication, December 8, 1944)

The appearance of *p*-hydroxyphenylpyruvic and lactic acids in the urine of scorbutic guinea pigs (1-3) and in the urine of premature infants who do not receive ascorbic acid (4) indicates that the body is unable to metabolize phenylalanine and tyrosine in the usual way in vitamin C deficiency. It has been shown (5-8) that liver slices from normal guinea pigs are able to (a) produce a hydroxyphenyl compound from phenylalanine, (b) metabolize tyrosine as measured by the disappearance of estimatable hydroxy groups, and (c) conjugate phenol. It was therefore of interest to determine whether these reactions occur to the same extent in liver slices from scorbutic guinea pigs. Lan and Sealock (9) have recently shown that liver tissue from deficient guinea pigs is unable to oxidize the side chain of tyrosine. The following experiments show that reactions involving the ring occur equally well *in vitro* in the normal and scorbutic liver.

## EXPERIMENTAL

Female guinea pigs from 250 to 350 gm. in weight were placed in individual cages and given the following scorbutogenic diet *ad libitum*: skim milk powder, previously heated at 110° for 2½ hours 300, rolled oats 390, wheat bran 200, cottonseed oil 80, cod liver oil 20, sodium chloride 10. This diet is a slight modification of that recommended by Sherman and Smith (10). The control guinea pigs were given a daily supplement by mouth of 5.0 mg. of crystalline ascorbic acid.

The animals on the unsupplemented basal diet developed signs of scurvy within 10 to 15 days, and these signs were corroborated by the autopsy findings. None of the control animals developed any sign of a deficiency. After each guinea pig developed scurvy it was placed in a metabolism cage and given orally 0.5 gm. of *L*-tyrosine. The urines were collected for the

\* Member of the Cooperative Nutrition Study, a joint project of the North Carolina State Board of Health, Duke University School of Medicine, and the International Health Division of the Rockefeller Foundation. Present address, Departments of Biochemistry and Medicine, School of Medicine, Vanderbilt University, Nashville.

next 24 hour period. In all cases the scorbutic guinea pigs were found to excrete an abnormal metabolite which gave a green color when the acidified urine was treated with a few drops of a 5 per cent solution of ferric chloride. None of the animals which received ascorbic acid excreted this metabolite, and the scorbutic guinea pigs excreted it only after the test dose of tyrosine.

After the demonstration of the *in vivo* occurrence of this defect in tyrosine metabolism in the deficient guinea pigs, they and their corresponding

TABLE I

*Production of Hydroxyphenyl Compound from 6.0 Mg. of dl-Phenylalanine and Disappearance of Estimable Hydroxy Groups from 1.0 Mg. of l-Tyrosine after Incubation with Normal and Scorbutic Guinea Pig Liver Slices for 4 Hours at 37°*

The scorbutic liver slices contained 5 per cent more dry weight than the normal.

Experiment No.	Hydroxyphenyl formed from phenylalanine		Hydroxy groups disappeared from tyrosine	
	Control	Scorbutic	Control	Scorbutic
	mg.	mg.	mg.	mg.
1	0.22	0.22		
2	0.29	0.37		
3	0.23	0.63	0.70	0.35
4	0.51	0.49	0.44	0.39
5	0.57	0.48	0.38	0.43
6	0.38	0.21	0.49	0.40
7	0.47	0.57	0.28	0.21
Mean.....	0.38	0.42	0.46	0.36

TABLE II

*Amount of Phenol Conjugated by Normal and Scorbutic Guinea Pig Liver Slices after Incubation with 0.04 Mg. of Phenol at 37° for 90 Minutes*

Experiment No.	Control	Scorbutic
	γ	γ
1	9.5	10.5
2	8.0	9.0

controls were killed by a blow on the head. The liver was removed and slices weighing approximately 300 mg. were incubated in 50 cc. Erlenmeyer flasks with 4.0 cc. of Krebs' bicarbonate solution for 4 hours at 37° in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide. 6.0 mg. of *dl*-phenylalanine or 1.0 mg. of *l*-tyrosine was added to certain of the flasks. Others contained liver slices only. To the latter at the end of the experiment different amounts of tyrosine were added, followed immediately

by 1.0 cc. of 20 per cent trichloroacetic acid. These were used as standards for estimating the amount of hydroxyphenyl compounds in the experimental flasks. After centrifuging, the estimations were carried out according to the method of Theis and Benedict (11). The results are shown in Table I. For the determination of the amount of phenol conjugated, the liver slices were incubated in Krebs' phosphate solution and air. 0.04 mg. of phenol was added to each flask, and after 90 minutes incubation the protein was precipitated by trichloroacetic acid and the amount of phenol remaining, before and after boiling with HCl, was estimated by the same method. The results are shown in Table II.

#### DISCUSSION

These results together with those of Lan and Sealock indicate that the main defect in the metabolism of aromatic compounds by scorbutic guinea pigs is the inability of the deficient liver to oxidize the side chain of tyrosine rather than an inability to oxidize the ring or conjugate the phenolic group. It is not known what happens to the side chain since there is no evidence that deamination occurs *in vitro*, but apparently ascorbic acid can be considered a part of the enzyme which catalyzes the oxidation. In this connection it is interesting that another vitamin, pyridoxine, is involved in the decarboxylation of tyrosine by *Streptococcus faecalis* R (12).

#### SUMMARY

Liver slices from scorbutic guinea pigs produce a hydroxyphenyl compound from phenylalanine, metabolize tyrosine as indicated by the disappearance of estimatable hydroxy groups, and conjugate phenol to the same extent as liver slices from normal guinea pigs.

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# ON THE MECHANISM OF THE CONVERSION OF ORNITHINE TO PROLINE IN VIVO; INTRAMOLECULAR NITROGEN SHIFT

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Krebs (1) has shown that *d*-proline and *d*-ornithine are interrelated, for they both yielded  $\alpha$ -keto- $\delta$ -aminovaleric acid on oxidation with *d*-amino acid oxidase. On the basis of this finding and their observation of the mutual interconversion of ornithine and proline (2, 3), Stetten and Schoenheimer proposed (2) that these amino acids are interconvertible through the intermediate,  $\alpha$ -keto- $\delta$ -aminovaleric acid.

It has also been demonstrated that on feeding amino acids labeled with  $N^{15}$  the ornithine as well as the amidine groups of arginine contained isotopic nitrogen (4). More recently it has been shown (2) that both the  $\alpha$ - and  $\delta$ -amino groups of ornithine contained a small amount of  $N^{15}$  after the ingestion of proline containing isotopic nitrogen, and the present authors (5) have demonstrated that, 4 days after the feeding of glycine containing isotopic nitrogen, the ornithine isolated from the carcass arginine contained the same concentration of  $N^{15}$  in the  $\alpha$ -amino and  $\delta$ -amino groups. This equality of isotope concentration may have been either fortuitous or indicative of a reaction mechanism. If it were of biological significance, the mechanism as proposed by Stetten and Schoenheimer for the interconversion of proline and ornithine would not be able to explain the result, for the equality of isotope concentration in the  $\alpha$ - and  $\delta$ -amino groups of ornithine could occur only if both nitrogens were interchangeable by some mechanism or were both derived from the same source. From the mechanism postulated by those authors, the  $\alpha$  position of ornithine can be reversibly deaminated and aminated by isotopic nitrogen originating from diverse sources, but the  $\delta$ -amino group would originate only from proline. In the presence of isotopic ammonia, the reversible amination and deamination of the  $\alpha$  group of ornithine would continuously change the isotope concentration of the  $\alpha$ -amino group without affecting the  $\delta$ -amino group.

In order to investigate this problem in greater detail, glycine containing isotopic nitrogen was incorporated into the diet of a number of rats for 3 days; the animals were killed in groups (during the ensuing week), 0, 4, and 7 days after feeding, and the arginine isolated from the pooled muscle and skin. The isotope concentration of the arginine, the amidine group,

the ornithine moiety, and the  $\alpha$ -amino group was determined at each period. The concentration of the  $\delta$ -amino group of ornithine at each period could then be calculated from the values of the isotope concentrations of the ornithine and the  $\alpha$ -amino group. It can be seen from Table I that, within the experimental error, in each case the  $\alpha$ -amino and  $\delta$ -amino groups of ornithine contained the same concentration of isotopic nitrogen.

This finding demonstrates that the  $\alpha$ -amino groups of arginine and of ornithine are not reversibly deaminated and aminated, for if the  $\alpha$ -amino group underwent deamination and reamination the isotope concentration of the  $\alpha$ -amino group would be different from that of the  $\delta$ -amino group. From the above finding another mechanism is proposed to explain the identity of isotopic nitrogen in the  $\alpha$ - and  $\delta$ -amino groups of ornithine and the conversion of ornithine to proline.

TABLE I

*Distribution of  $N^{15}$  in Arginine Isolated from Rat Carcasses*

The values are given in atom per cent excess  $N^{15}$ .

Group No.	$N^{15}$ concentrations in					
	Arginine	Amidine N	Ornithine (found)	Ornithine* (calculated)	$\alpha$ -Nitrogen	$\delta$ Nitrogen†
0	0.070	0.106	0.031	0.034	0.034	0.028
4	0.109	0.157	0.063	0.061	0.064	0.062
7	0.147	0.204	0.087	0.090	0.090	0.084

\* These values are calculated from the isotope concentrations of the arginine and the amidine nitrogen.  $C_{\text{ornithine}} = 2C_{\text{arginine}} - C_{\text{amidine}}$ .

† These values are calculated from the isotope concentrations of the ornithine and the  $\alpha$ -nitrogen.  $C_{\delta\text{-N}} = 2C_{\text{ornithine}} - C_{\alpha\text{-N}}$

#### EXPERIMENTAL

The experiments reported here were made with the arginine samples isolated from the rat carcasses (muscle and skin) of a previous experiment (5). Into the low nitrogen stock diet of each rat, 146 mg. of isotopic glycine (34.4 atom per cent excess  $N^{15}$ ) were incorporated each day for 3 days. After the rats had received the glycine for 3 days, three of the rats were killed (Group 0). The remaining rats were then kept on the stock diet. 4 days later, two rats (Group 4), and finally 7 days later the remaining three rats were killed (Group 7). From the combined protein fractions of the carcasses of each group arginine was isolated as the flavianate. The arginine samples of Groups 0 and 7 were purified through the hydrochloride and that of Group 4 was purified as the *p*-toluenesulfonyl derivative. The  $N^{15}$  concentrations of the arginine samples were determined (Table I).

*Distribution of N<sup>15</sup> in Arginine*

*N<sup>15</sup> Concentration of Amidine Group of Arginine*—The arginine monohydrochloride and the  $\alpha$ -*p*-toluenesulfonylarginine samples were refluxed with a saturated Ba(OH)<sub>2</sub> solution for 24 hours and the liberated ammonia swept into a dilute sulfuric acid trap by a stream of nitrogen. The N<sup>15</sup> concentration of the amidine nitrogen was then determined (Table I).

*N<sup>15</sup> Concentration of Ornithine Moiety of Arginine*—The ornithine formed in the barium hydroxide solution was isolated as the dibenzoyl derivative from samples of Group 0 and Group 7; that of Group 4, as the  $\alpha$ -*p*-toluenesulfonyl derivative. The N<sup>15</sup> concentrations of these samples were determined (Table I).

*N<sup>15</sup> Concentration of  $\alpha$ -Nitrogen of Arginine*—The arginine samples of Groups 0 and 7 were treated with ninhydrin as described by MacFadyen (6) and the liberated  $\alpha$ -nitrogen was analyzed for its isotope concentration (Table I). The N<sup>15</sup> concentration (Table I) of the  $\alpha$ -nitrogen of arginine of Group 4 was determined by analyzing the amide of  $\alpha$ -toluenesulfonyl-amino- $\delta$ -hydroxyvaleric acid (5).

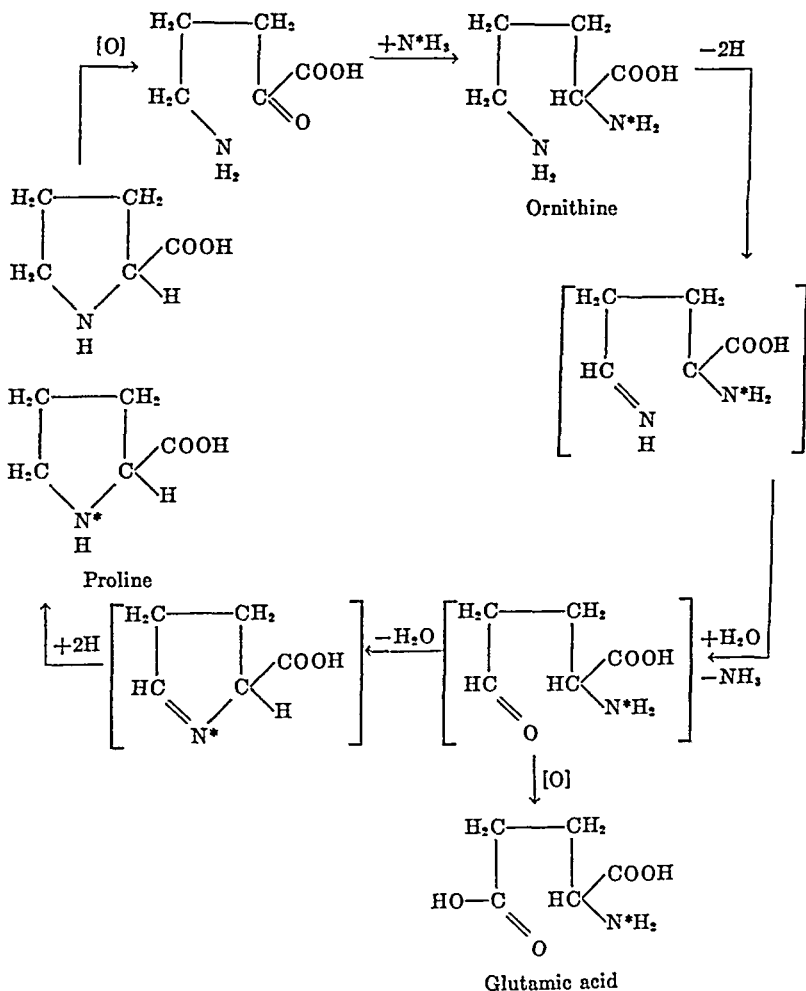
## DISCUSSION

The experimental finding that the  $\alpha$ - and  $\delta$ -amino groups of ornithine contain the same concentration of isotope after the feeding of glycine containing N<sup>15</sup> demonstrates the probability that the nitrogen atoms which form both these groups have a common source. A discussion of three possible mechanisms follows.

As suggested by Stetten and Schoenheimer (2), proline may be oxidized between the nitrogen atom and either the  $\alpha$ - or  $\delta$ -carbon atoms. The respective products would be  $\alpha$ -keto- $\delta$ -aminovaleric acid and the semialdehyde of glutamic acid. It is conceivable that the latter acid, as well as the former, could be aminated by nitrogen originating from other amino acids to form ornithine. This mechanism would necessitate the improbable assumption that the over-all rates of deamination and reamination of the semialdehyde of glutamic acid and the  $\alpha$ -keto- $\delta$ -aminovaleric acid are the same. Moreover, on oxidation of *d*- or *l*-proline with *d*- and *l*-amino acid oxidase (1, 7) the keto acid was the only one isolated.

It is possible that both the  $\alpha$ - and  $\delta$ -amino groups of ornithine are reversibly removed and replaced with nitrogen from other amino acids. This scheme presupposes that the process proceeds at identical rates at both the  $\alpha$ - and the  $\delta$ -carbon atoms. Such a coincidence seems unlikely. Moreover, it appears doubtful whether the  $\alpha$  group of *l*-ornithine is deaminated at a significant rate. Krebs (1) has shown that *d*-ornithine is deaminated by kidney extract to form  $\alpha$ -keto- $\delta$ -aminovaleric acid, but the reaction was

very slow and the formation of this ketonic acid from *d*-ornithine took place with one-fortieth the rate of formation of the ketonic acid from *d*-proline. The formation of  $\alpha$ -keto- $\delta$ -aminovaleric acid from *l*-ornithine



$\text{N}^*$  = isotopic nitrogen.

has not been demonstrated. An *l*-amino acid oxidase has recently been isolated which deaminates most of the monoaminomonocarboxylic acids but attacks neither lysine, arginine, nor ornithine (7).

A third mechanism, which would explain the equality of the isotope concentration of the  $\alpha$ - and  $\delta$ -amino groups of ornithine and incidentally also explain the mechanism of the conversion of ornithine to proline, may be postulated as in the accompanying diagram. Proline is oxidized by *L*-amino acid oxidase to  $\alpha$ -keto- $\delta$ -aminovaleric acid (7); the nitrogen originating from the proline is now in the  $\delta$  position. This keto acid is then irreversibly aminated in the  $\alpha$  position by ammonia to form ornithine. The  $\alpha$ -amino group originates from nitrogen of amino acids other than proline. The ornithine thus formed is converted to proline by the over-all splitting out of ammonia from the  $\delta$ -amino group only. This step may be postulated to involve dehydrogenation at the  $\delta$  position, with production of  $\alpha$ -amino- $\delta$ -iminovaleric acid. This hypothetical intermediate would by hydrolysis yield glutamic semialdehyde, which could, by further oxidation, yield glutamic acid (3), or, by spontaneous ring closure, yield pyrroline-carboxylic acid. This should be readily convertible by hydrogenation *in vivo* into proline. Such a conversion of ornithine to proline would involve the loss of the  $\delta$ -nitrogen and not the  $\alpha$ -nitrogen, and the proline would contain the same nitrogen which was the  $\alpha$ -nitrogen in the ornithine. At this point this nitrogen of proline is shifted to the  $\delta$  position by oxidation of the proline to form the  $\alpha$ -keto- $\delta$ -aminovaleric acid. On amination of the keto acid to form ornithine a new nitrogen atom enters the  $\alpha$  position. By this cyclic process every nitrogen atom in the  $\delta$  position passes through the  $\alpha$  position.

This proposed mechanism, involving an intramolecular nitrogen shift, is attractive, for it explains best the equality of isotopic concentration in the  $\alpha$ - and  $\delta$ -amino groups of ornithine after the feeding of glycine containing isotopic nitrogen without the necessity of postulating the improbable equality of rates of amination and deamination on the  $\alpha$ - and  $\delta$ -carbons of ornithine.

The equality of isotopic nitrogen in the  $\alpha$ - and  $\delta$ -amino groups in ornithine definitely demonstrates that arginine is not reversibly deaminated and aminated as such. One can draw this definite conclusion, for in arginine the  $\delta$ -nitrogen is linked to the amidine group and if the  $\alpha$ -nitrogen of arginine were able to undergo this reversible process the isotopic concentration of the  $\alpha$ - and  $\delta$ -nitrogen atoms of ornithine would differ greatly.

#### SUMMARY

The feeding of glycine, containing isotopic nitrogen, to rats results in the incorporation of isotopic nitrogen of equal concentration in the  $\alpha$ - and  $\delta$ -amino groups of ornithine. A mechanism involving an intramolecular nitrogen shift is proposed to explain this finding and for the conversion of ornithine to proline. Also from the above result the conclusion may be

drawn that the  $\alpha$ -amino group of arginine is not reversibly deaminated and aminated and this is probably true as well for the  $\alpha$ -amino group of ornithine.

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# THE METABOLISM OF TRIMETHYLACETIC (PIVALIC) AND TERTIARY BUTYLACETIC ACID. NEW EXAMPLES OF CONJUGATION WITH GLUCURONIC ACID\*

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In unpublished experiments from this laboratory, young white rats were fed trimethylacetic (pivalic) acid and tertiary butylacetic acid (as the sodium salts) in amounts ranging from 25 to 250 mg. per 100 gm. of body weight. No toxicity was observed either with single administrations or when the feedings (25 to 100 mg. of the acids per 100 gm. of body weight) were continued daily over periods of 7 to 14 days. The salts of these acids were relatively non-toxic to rabbits also.

Examination of the urines excreted in these experiments showed frequently a notable rise in urinary nitrogen and a heightened "creatinine" excretion on the experimental days. When the urines were treated with picric acid and sodium hydroxide (Jaffe test), the yellow color, which is measured in the usual method for the determination of creatinine, became more intense as the reaction mixture was allowed to stand. This suggested the presence of some substance, which gave a positive Jaffe test, other than creatinine. Glucuronic acid has been reported to give a color in this reaction, a color which increases in intensity as the mixture stands (1). Positive tests with the Tollens naphthoresorcinol and Benedict's sugar reagents were also observed, tests which indicated the presence of glucuronic acid.

These preliminary observations led us to a more careful study of the metabolism of these two aliphatic acids. The present investigation is unique in that, so far as is known to us, it is the first in which the metabolism of an aliphatic fatty acid containing a tertiary carbon atom has been studied. We have been able to show that the ingestion of these acids (as the sodium salts) is followed by a significant excretion of glucuronic acid in the urine, in amounts which are equivalent to a very considerable percentage of the ingested aliphatic acid. While it has not been possible to isolate a conjugated glucuronic acid, all the evidence supports the hypothesis that the acids are not readily oxidized but are excreted in large part as conjugated glucuronides. It has long been known that certain aromatic

\* We wish to express our appreciation to the Mallinckrodt Chemical Works of St. Louis for a grant in support of this work and also for the gift of the trimethylacetic and tertiary butylacetic acids used in the experiments.



acids, as benzoic and phenylacetic, may be conjugated with glucuronic acid in the animal organism (2). The conjugation of an *aliphatic acid* with glucuronic acid has not been reported previously, to our knowledge.

#### EXPERIMENTAL

Young male rabbits, 2 to 4 kilos in weight, housed in metabolism cages which permitted the quantitative collection of 24 hour specimens of urine, were maintained on uniform diets of either a commercial rabbit chow or oats and cabbage. The compounds studied were administered in aqueous solution through a stomach tube or injected subcutaneously at the beginning of the experimental period.

The trimethylacetic or pivalic (3) and tertiary butylacetic acids were made available to us by the courtesy of the Mallinckrodt Chemical Works of St. Louis. Inactive trimethylsuccinic acid ( $\alpha$ -hydroxy- $\beta,\beta$ -dimethylbutyric acid) was prepared in this laboratory. Tertiary butylacetic acid was brominated (Hell-Volhard-Zelinsky reaction) to yield  $\alpha$ -bromo- $\beta,\beta$ -dimethylbutyric acid. This acid was dissolved in water, was neutralized with 0.1 N sodium hydroxide, and the solution was refluxed on the steam bath for 24 to 36 hours. The solution was then made definitely acid to Congo red (10 per cent sulfuric acid), and extracted repeatedly with small portions of ether. The ether was allowed to evaporate at room temperature. The residual syrup crystallized on standing. The crude product was dissolved in water and the solution was decolorized with norit. The white crystals which separated on concentration of the filtrate were dried at room temperature over phosphorus pentoxide. The acid melted at 87–88° (uncorrected) and, on analysis, the following values were obtained.

	Found per cent	Theory per cent
C.....	54.16, 54.36	54.54
H.....	9.14, 9.19	9.09
Neutral equivalent.....	132	132

So far as is known to us, the acid has not been prepared previously by this procedure.

For the analyses of the urines, standard procedures were utilized: modified Kjeldahl (total nitrogen), Folin (creatinine), and Folin and Benedict-Denis (partition of urinary sulfur).

The concentration of glucuronic acid in the urine was determined essentially by the procedure of Maughan, Evelyn, and Browne (4). The specificity of the Tollens naphthoresorcinol reaction on which this procedure is based has been questioned (5–8), but it is usually agreed that the method is applicable to a dilute fluid such as urine in the absence of any considerable amounts of reducing sugars (4, 6, 8, 9). All the "glucuronic acid"

as determined in normal rabbit urine by this method may not be true glucuronic acid.<sup>1</sup> We believe, however, that when a significantly increased content is observed in urines after the administration of compounds which are known to conjugate with glucuronic acid these values represent increases in true glucuronic acid, and that the calculated "extra" glucuronic acid gives an accurate measure of the amount of the foreign compound thus conjugated.

Since it was not possible to secure adequate amounts of naphthoresorcinol for use in the determination, the synthesis of this reagent (10) was attempted. The reactions proceeded smoothly up to the last step, but the final reaction, the conversion of 1,3-dihydroxynaphthalene-2-carboxylic acid to naphthoresorcinol, failed to give satisfactory yields. It was observed that good colors could be obtained in the Tollens reaction as applied quantitatively, if an aqueous solution of the acid were used in place of naphthoresorcinol. Others have used the acid but have decarboxylated the compound in solution prior to the reaction with uronic acid (11, 12). In our work, we have substituted for the 0.2 per cent naphthoresorcinol solution usually recommended an aqueous 0.2 per cent solution of the 1,3-dihydroxynaphthalene-2-carboxylic acid.<sup>2</sup> All results are expressed in terms of D-glucurone, which was used to determine the standard *K* values and was also employed in experiments to determine whether glucurone added to urine could be satisfactorily recovered. D-Glucurone<sup>3</sup> was prepared by the hydrolysis of the ammonium salt of menthylglucuronic acid (isolated from the urine of rabbits to which menthol had been fed) according to the procedure described by Williams (13). When solutions containing from 40 to 100  $\gamma$  of this pure glucurone per 2 ml. were subjected to the standard analytical procedure, uniform *K* values were obtained (average,  $320 \times 10^{-5}$ ). These values were checked repeatedly throughout the course of the experiments with the use of pure glucurone.

The administration of the sodium salt of trimethylacetic or tertiary butylacetic acid, either orally or subcutaneously, was followed by the excretion of extra glucuronic acid in the urine. Details of three typical ex-

<sup>1</sup> After the completion of the present work, Hanson, Mills, and Williams (9) reported an average daily glucuronic acid excretion by normal rabbits of 140 to 150 mg. with a range of 56 to 218 mg. These "normal" values are similar to our own (cf. Tables I and II).

<sup>2</sup> Maughan, Evelyn, and Browne (4) have recommended the use of a filter which transmits light in the region of 565  $m\mu$  in the Evelyn photoelectric colorimeter. We have found a 540  $m\mu$  filter satisfactory. Full details of the colorimetric procedure as employed by us are available in the doctoral dissertation of Dominic D. Dziewiatkowski on file in the library of the University of Michigan.

<sup>3</sup> We are indebted to Dr. Walther F. Goebel of The Rockefeller Institute for Medical Research for a sample of pure glucurone, which served as a standard of comparison with the product prepared by us.

periments with trimethylacetic acid are presented in Table I. The excretion of extra glucuronic acid continued for 48 to 72 hours after the administration of the salt of the acid. The urines which contained the extra glucuronic acid all reduced Benedict's copper reagent for sugars. It

TABLE I  
*Excretion of Glucuronic Acid after Administration of Sodium Salt of Trimethylacetic Acid*

In the last two columns the amount of acid conjugated is calculated on the assumption that all of the extra glucuronic acid is conjugated mole per mole with the aliphatic acid fed. Per cent refers to the percentage of the amount administered thus conjugated. Glucuronic acid is calculated as glucurone.

Experiment No.	Creatinine	Nitrogen	Glucuronic acid		Acid conjugated	
			Total	Extra		
	mg.	gm.	mg.	mg.	mg.	per cent
2-1	150	1.81	116			
	149	1.69	110			
	163	2.25	625*	512	297	36
	161	1.74	216	103	58	7
	153	1.76	143†	30	17	2
	151	1.64	149			
	156	1.39	99			
3-4	105	0.64	70			
	113	0.71	318‡	248	144	35
	116	0.74	287	217	125	31
	98	0.56	83			
	119	0.64	75			
12-4	215	1.98	175			
	247	2.12	702*	527	306	37
	(65)§	(0.42)	(226)	(175)		(12)
	(182)§	(1.70)	(476)	(352)		(25)
	251	2.83	256	81	47	6
	192	1.66	100			

\* 1.0 gm. of the sodium salt of trimethylacetic acid was fed.

† The urine reduced Benedict's reagent on this day; no reduction was observed on the following day despite the high value for glucuronic acid.

‡ 0.5 gm. of the sodium salt of trimethylacetic acid was injected subcutaneously.

§ The values of these two horizontal columns represent the excretions in the 6 and 18 hour periods which together comprise the 24 hour period of the horizontal column immediately preceding.

will be noted that, in Experiment 2-1, the urine of the 4th day after feeding contained 149 mg. of glucuronic acid, an amount somewhat greater than the usual control values. However, since no reduction with Benedict's reagent was observed, this is not believed to represent true glucuronic acid.

In all cases in which extra glucuronic acid was calculated as shown in Tables I and II, the urines gave strongly positive Benedict's tests and also contained glucuronic acid in amounts definitely greater than normal.

The results of the administration of the salt of tertiary butylacetic acid were, with one exception, similar to those just discussed (Table II). The excretion of extra glucuronic acid was complete in the first 24 hours after

TABLE II

*Excretion of Glucuronic Acid after Administration of Sodium Salt of Tertiary Butylacetic Acid*

For the explanation of Table II, the heading of Table I should be consulted.

Experiment No.	Creatinine	Nitrogen	Glucuronic acid		Acid conjugated	
			Total	Extra		
	mg.	gm.	mg.	mg.	mg.	per cent
12-1	222	2.06	119			
	220	2.11	119			
	220	2.43	840*	721	476	57
	215	1.93	122			
	220	1.92	138			
	220	1.95	122			
	218	1.98	121			
13-1	154	0.82	82			
	167	0.81	61			
	157	0.85	648†	577	380	90
	172	0.76	84			
	153	0.61	50			
13-2	135	0.61	67			
	185	0.93	943*	876	575	69
	(65)‡	(0.25)	(418)	(392)		(31)
	(120)‡	(0.68)	(525)	(484)		(38)
	156	0.80	68			
	138	0.74	53			

\* 1 gm. of the sodium salt of tertiary butylacetic acid was fed.

† 0.5 gm. of the sodium salt of tertiary butylacetic acid was injected subcutaneously.

‡ The values of these two horizontal columns represent the excretions in the 6 and 18 hour periods which together comprise the 24 hour period of the horizontal column immediately preceding.

the administration of the salt of the acid. This difference in the rate of excretion is difficult to explain, since the two acids differ only by one methylene ( $-\text{CH}_2-$ ) group in the length of the chain.<sup>4</sup> That the mobilization of the glucuronic acid occurs rapidly is shown by the data of Experi-

<sup>4</sup> Carter (14) has also reported differences in the biological behavior of certain fatty acids with branched chains which differ from each other by only a methylene group.

ments 12-4 (Table I) and 13-2 (Table II) in which the 24 hour urine was analyzed in two fractions. In the specimens collected in the first 6 hours, a significant amount of extra glucuronic acid was present.

Table III summarizes all the experiments with the two acids. On the assumption that all the extra glucuronic acid is conjugated mole for mole with the acid, we have calculated the percentage of the aliphatic acid fed which is excreted in conjunction with the glucuronic acid; *i.e.*, 43 to 75 and

TABLE III

*Summary of Experiments with Trimethylacetic and Tertiary Butylacetic Acids*

1 gm. of the sodium salt of the acid was administered orally, except in Experiments 3-3, 3-4, and 13-1, in which 0.25, 0.5, and 0.5 gm. respectively were injected subcutaneously. For further explanations of this table, the heading of Table I should be consulted.

Acid	Experiment No.	Extra glucuronic acid excreted	Acid conjugated	
		mg.	mg.	per cent
Trimethylacetic.....	2-1	645	372	45
“.....	3-1	1065	612	75
“.....	3-2*	993	574	70
“.....	3-3†	304	175	86
“.....	3-4†	465	269	66
“.....	3-5	747	433	53
“.....	12-2	886	512	62
“.....	12-4	608	353	43
Tertiary butylacetic.....	2-2	849	559	66
“.....	2-3*	670	441	52
“.....	2-5	671	442	53
“.....	4-1	868	572	68
“.....	12-1	721	476	57
“.....	13-1†	577	380	90
“.....	13-2	876	575	69

\* The animal was fasted for 3 days prior to the experimental day and during the experimental day.

† Subcutaneous administration.

52 to 68 per cent after feeding trimethylacetic and tertiary butylacetic acids, respectively. The slightly higher values in two of the three experiments in which subcutaneous administration was employed (Experiments 3-3, 3-4, and 13-1, Table III) may be explained by the smaller amounts of the acids injected in these experiments.

The isolation of conjugated glucuronic acids from the experimental urines by modifications of the procedures usually employed in such studies (14) has as yet been unsuccessful. The ease with which the urines reduced

Benedict's reagent without previous hydrolysis suggested that we were concerned with a labile glucuronide, comparable to the conjugation product of the aromatic organic acid, benzoic, *i.e.* benzoylglucuronide, the isolation and proof of structure of which presented difficulty in early studies (15-17).

An attempt was made to isolate and identify the unchanged acids, presumably derived from the hydrolysis of the urinary glucuronates. An aliquot of the urine was acidified with 85 per cent phosphoric acid (indicator, Congo red paper), an extra 2 ml. of the acid were added, and the acidified urine was distilled<sup>5</sup> from a small Claisen flask. The distillate was collected in a flask cooled with an ice-salt mixture, and the distillation was continued until only a thick gummy residue remained. The distillate was neutralized (phenolphthalein indicator) with standard alkali by titration, the neutralized solution was evaporated to dryness on a steam bath, and the residue was dissolved in the minimum amount of hot water. The hot solution was added with stirring to a volume of hot, 15 per cent alcoholic solution of S-benzylthiuronium chloride,<sup>6</sup> an amount which was only slightly in excess of that required for the reaction with the organic acid (18). The amount necessary was calculated from the volume of standard alkali used for neutralization of the distillate. White crystals separated on cooling, which, after recrystallization from a mixture of equal parts of alcohol and water, melted satisfactorily. The melting points and analyses of the S-benzylthiuronium salts prepared from the pure acids and from the distillates of the urine as described are given in Table IV. These studies have thus demonstrated that, in part at least, the acids had escaped oxidation and could be isolated from the urine.

It was not anticipated that a quantitative recovery of the aliphatic acids from the urine as the S-benzylthiuronium salts would be possible. In two experiments in which 1.93 and 1.42 mm of trimethylacetic acid were added to rabbit urine, it was possible to recover as the salt 48.5 and 59.1 per cent, respectively, of the amounts added. In similar analyses in which 0.33 and 2.55 mm of tertiary butylacetic acid were added, recoveries of 46.5 and 71.2 per cent were obtained. A comparison of the amount of extra glucuronic acid excreted and the amount of the S-benzylthiuronium salt isolated from the same urine was made (Table V). If the amount of acid isolated as the salt were significantly greater than the amount calculated to be in combination with glucuronic acid, this would be convincing evidence for the excretion of the aliphatic acid in a form other than as the glucuronide.

<sup>5</sup> The boiling points of trimethylacetic and tertiary butylacetic acids are approximately 164° and 181°, respectively.

<sup>6</sup> We are indebted to Dr. Stanley Levey of this laboratory for advice in the preparation of the S-benzylthiuronium salts. This reagent has been found to be of significant value in the isolation of organic acids from biological materials in this laboratory.

If, on the other hand, the amounts thus calculated should be similar (the quantitative error in the isolation of the S-benzylthiuronium salt being taken into consideration), this would suggest that the acid was excreted as a glucuronide. Such a comparison is presented in Table V for four experiments with rabbits and two with rats. The data are not inconsistent with the existence in the urine of conjugated glucuronides (or glucuronates),

TABLE IV

*Melting Points and Analyses of S-Benzylthiuronium Salts of Trimethylacetic and Tertiary Butylacetic Acids*

All derivatives were prepared from rabbit urine unless otherwise indicated. The melting points are uncorrected. The mixed melting points of the derivatives of the pure acids and those isolated from the urines were satisfactory.

The calculated analyses for the S-benzylthiuronium salt of trimethylacetic acid,  $C_{11}H_{20}O_2N_2S$ , are carbon 58.20 per cent, hydrogen 7.46 per cent, and nitrogen 10.44 per cent. For the similar salt of tertiary butylacetic acid,  $C_{14}H_{22}O_2N_2S$ , the calculated values are carbon 59.57 per cent, hydrogen 7.80 per cent, and nitrogen 9.92 per cent.

Acid	Sample No.	M.p.	Nitrogen	Carbon	Hydrogen
		°C.	per cent	per cent	per cent
Trimethylacetic.....	1*	153	10.45	58.23	7.28
“ .....	5	152-153	10.34		
“ .....	10	153	10.03		
“ .....	13†	152-153	10.30	57.36	7.23
“ .....	14†	152-153	10.42	57.65	7.21
“ .....	2R†	153	10.41		
Tertiary butylacetic.....	2*	165	9.90	59.84	7.73
“ .....	6	164-165	9.60		
“ .....	7	164-165	9.88	59.31	7.76
“ .....	8	164-165	9.68		
“ .....	12	164-165	9.86		
“ .....	1R†	164-165	9.57		

\* Derivatives of the pure acids.

† These salts were not completely dried; an analysis of Sample 1, dried under the same conditions as were these samples, gave values of 57.57 and 7.39 per cent of carbon and hydrogen, respectively.

‡ Isolated from rat urine.

which must be of the labile type, similar to benzoylglucuronide, since the urines without previous hydrolysis reduced Benedict's sugar reagent. It was necessary to interrupt this study before the isolation of the conjugated compound was achieved. It is hoped that the study of the problem may be continued at a later date.

The qualitative evidence of the presence of glucuronic acid in the experi-

mental urines was supported by the preparation of the 2,4-dinitrophenylhydrazide of glucuronic acid by slight modifications of the procedure described by Williams (13). The derivative thus obtained was compared with the similar derivatives prepared from a sample of glucurone obtained from Dr. Walther F. Goebel and a second sample prepared by hydrolysis of menthylglucuronic acid in our own laboratory. All the hydrazides melted at 199–200° (uncorrected) with decomposition and the melting points were not depressed when the hydrazides of various origins were mixed. Williams has reported a melting point of 205°. The nitrogen content of four samples of the hydrazide prepared from urine varied from 14.67 to 14.87 per cent (theory, 15.00 per cent).

TABLE V

*Comparison of Amount of Extra Glucuronic Acid Excreted with Amount of S-Benzylthiuronium Salt Isolated from Urines*

Unless otherwise indicated, the experimental animals were rabbits.

Acid fed		Extra glucuronic acid in 24 hrs. (a)	Salt isolated		
			(b)	(b/a) × 100	Corrected*
	mM	mM	mM	per cent	per cent
Trimethylacetic	16.10	5.93	2.09	35.3	63+
	16.10	8.43	2.95	35.0	63—
	4.00†	2.20	0.98	44.1	79—
Tertiary butylacetic	14.40	10.50	3.60	34.2	61+
	28.80	25.90	12.45	48.1	86—
	2.97†	1.93	0.89	46.6	83+

\* Corrected from the figures in the preceding column, on the assumption that the amount of the derivative actually recovered represents 56 per cent (average of four recovery experiments described in the text) of the acid actually present.

† Rat experiment.

The possibility that these aliphatic acids may also be excreted in conjugation with glycine has not been investigated. Many aromatic acids, not easily oxidized, have been shown to be excreted as conjugates of glycine. Although it seemed improbable that the acids whose behavior we were investigating or products of their metabolism would be conjugated with sulfuric acid or with cystine (as is monobromobenzene), the distribution of urinary sulfur was determined in the urine of rabbits fed each of the acids under consideration. No changes in the distribution of sulfur were observed.

In a continuation of the study of aliphatic acids in whose molecule a tertiary carbon atom was present, the sodium salt of  $\alpha$ -hydroxy- $\beta,\beta$ -dimethylbutyric acid (trimethylacetic acid) was fed to two rabbits in an



amount equivalent to 1 gm. of the salt of tertiary butylacetic acid (7.24 mm). The urines excreted in the 24 hour period immediately following administration of the acid reduced Benedict's reagent, gave a positive Tollens test, and on quantitative analysis were shown to contain extra glucuronic acid in amounts which were significant, although less than those observed in the experiments with the related tertiary butylacetic acid. Thus 167 and 287 mg. of extra glucuronic acid were present, equivalent to 13 and 22 per cent of the acid fed respectively. The excretion of extra glucuronic acid was completed in the first 24 hour period. A substance could be extracted from the acidified urine by ether, which gave the yellow color with Uffelmann's reagent characteristic of lactic acid and other  $\alpha$ -hydroxy acids.

#### DISCUSSION

The recovery of significant amounts of fatty acids from the urine after the administration of the two aliphatic acids which contain tertiary carbon atoms was unexpected. It has been stated that "fatty acids with branched chains readily undergo complete oxidation" and that "within certain natural limitations Knoop's hypothesis of  $\beta$ -oxidation is applicable to the case of fatty acids with branched chains" (19). These conclusions have been drawn, however, from the study of acids which contain, in the  $\alpha$  or  $\beta$  position, a secondary carbon atom ( $=CH-$ ) rather than a tertiary carbon atom ( $\equiv C-$ ), which is present in the compounds here investigated. The isomers of trimethylacetic acid,  $\alpha$ - and  $\beta$ -methylbutyric acid, and of tertiary butylacetic acid,  $\alpha$ - and  $\beta$ -ethylbutyric acid, have been shown to have a biological behavior similar to that of butyric acid (19). However, in these isomeric acids, both the  $\alpha$ - and  $\beta$ -carbon atoms still have at least 1 hydrogen atom attached and it is assumed that  $\alpha$  or  $\beta$  oxidation or an  $\alpha, \beta$  desaturation may occur as the initial reaction in the oxidation (14). Since no hydrogen is attached to the  $\alpha$ - (trimethylacetic acid) or the  $\beta$ - (tertiary butylacetic acid) carbon atom of the acids which we have studied, the above reactions may presumably not be possible and the oxidation of the acids does not occur readily. The amounts of the acids recovered from the urine as the S-benzylthiuronium salts ranged from 35 to 50 per cent of the amount fed. If, as suggested by the control recovery experiments with rabbit urine, these values represent about half of the acid present in the urine samples, it is apparent that a relatively large fraction of the acid was excreted in the urine without oxidation. The isolation studies gave no indication of the presence of other volatile acids which might have been formed. The ease with which the S-benzylthiuronium salts were purified speaks against the presence in significant amounts of any *volatile* acid other than the one actually isolated. The possibility that *non-volatile* hydroxy acids were present in the urines is not ruled out.

Although the isolation of the conjugated glucuronide has not been accomplished, all the evidence points to the fact that the acids containing a tertiary carbon atom are not readily oxidized, but are excreted largely in conjugation with glucuronic acid.

In most experiments, the ingestion of the acids was followed by an increase in urinary nitrogen (rats, rabbits). It is difficult to interpret such changes in urinary nitrogen, particularly with rabbits, in which the urinary nitrogen may vary somewhat irregularly. An increased protein metabolism, associated with the endogenous origin of the glucuronic acid, is suggested. This aspect of the problem will be considered more in detail in a subsequent communication, in which the biological origin of the glucuronic acid made available for conjugation will be discussed.

#### SUMMARY

The biological behavior of simple aliphatic acids which contain a tertiary carbon atom, trimethylacetic (pivalic) and tertiary butylacetic acids, after administration (oral and subcutaneous) to rats and rabbits has been studied.

Evidence is presented which indicates that the acids are not readily oxidized but are excreted in large part as labile conjugated glucuronides. This is believed to be the first reported instance of the conjugation of glucuronic acid with an *aliphatic* fatty acid.

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# POLYSACCHARIDE PRODUCTION BY VIRULENT AND ATTENUATED CROWN-GALL BACTERIA\*

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Because of the pathological growth induced by the crown-gall bacterium, *Phytomonas tumefaciens* (Smith and Townsend) Bergey *et al.*, the metabolic products of this organism are of special interest. Any differences between metabolic products from virulent and attenuated strains of crown-gall bacteria would receive particular attention because of their possible relation to diseased growth. In previous studies of these metabolites, McIntire, Peterson, and Riker (22) isolated and characterized a polysaccharide which accounted for 15 to 20 per cent of the sucrose fermented by a virulent strain of the organism. This polysaccharide was a water-soluble glucosan having a molecular weight of about 3600 and a specific rotation,  $[\alpha]_D^{20}$ , of  $-9^\circ$  to  $-10^\circ$ . The question arose whether an attenuated strain formed a polysaccharide, and if so whether it would be the same as that produced by the virulent organism. It was also desirable to know whether crown-gall bacteria formed glucosan from other sugars in addition to sucrose. It has been found that an attenuated strain produces a polysaccharide and that the virulent organism forms polysaccharide from various sugars. The preparation and properties of these metabolites are described below.

## EXPERIMENTAL

*Culture and Medium*—Two strains of *Phytomonas tumefaciens* were employed. Both the virulent strain, A6 (32), and the attenuated daughter strain, A6-6 (14), were progenies of single cells. Pathogenicities of these cultures were checked at intervals by inoculation into tomato plants. In all tests the virulent strain induced galls while the attenuated strain produced only rudimentary or no galls. The sucrose-mineral salts medium and the methods employed in growing the bacteria were the same as those described by McIntire *et al.* (22), except that in some fermentations either fructose, glucose, arabinose, or xylose was substituted for sucrose. All sugars were employed at 2 per cent concentrations. Except when otherwise indicated, all cultures were grown by inoculating 200 ml. of the medium

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with 2.5 per cent of a 24 hour liquid culture and incubating at  $26^{\circ} (\pm 0.5^{\circ})$  for 5 days with mechanical shaking, and the cultures were carried in 1 liter Erlenmeyer flasks which were provided with 6 inch extension necks to reduce splashing. In order to provide increased and uniform aeration, the cotton plugs were replaced by double layers of six ply, oil-treated, air-filter tissue.

*Analytical Methods*.—Carbon analyses were made by the wet oxidation method of Heck (13). Total reducing sugar was determined after hydrolysis according to the method of Shaffer and Somogyi (Reagent 50 with 5 gm. of potassium iodide per liter (30)). The nitrogen contents were estimated by a micromethod described by Johnson (18). Optical rotations were measured at approximately  $20^{\circ}$  with a Schmidt and Haensch polarimeter No. 52B, with monochromatic light. Samples for analyses were ground fine and dried to constant weight at approximately  $50^{\circ}$  over calcium chloride in a vacuum desiccator.

*Carbon Balance*.—The distribution of certain metabolites in fermented cultures of both virulent and attenuated strains was determined as follows. Parallel fermentations with sucrose as the chief carbon source were run according to the techniques already described. Several fermented cultures of each organism were combined, and each lot was fractionated as described below. The total volume of cultures handled varied from 1.5 to 7 liters. The pH of fermented cultures varied from 5.3 to 5.7. 10 ml. portions titrated to the color change of bromothymol blue required 0.7 to 0.8 ml. of 0.1 N sodium hydroxide. The fractionation was carried out without adjustments of the pH. After the cells were removed in a Sharples supercentrifuge, the clear supernatant liquid was treated with 10 ml. of saturated barium acetate solution per liter of culture and a precipitate (mostly inorganic) was allowed to settle for 1 to 2 hours before it was collected by centrifugation. The supernatant solution was concentrated by distillation under reduced pressure to about one-twentieth to one-thirtieth of the original volume, and an equal volume of ethyl alcohol was added. A precipitate formed and quickly settled. This precipitated fraction was designated as "gum" and was earlier studied by Conner *et al.* (6). This precipitate was removed by centrifuging and the clear solution was concentrated to about one-half its volume, and then poured dropwise with mechanical stirring into 9 to 10 volumes of absolute ethyl alcohol. A copious white precipitate of the polysaccharide studied separated at this point. After it had stood at room temperature for 1 to 2 hours, this was collected by centrifugation and the supernatant solution was treated with basic lead acetate. The precipitate that formed was removed by centrifuging and the final solution, designated as Filtrate D, was saved for analyses of total carbon and unfermented sucrose.

Analyses for total carbon and residual sucrose were made on the main solution before and after the separation of each fraction. The differences between the carbon in unfermented sucrose and total carbon at each point represented the metabolized carbon in the solution. Differences between metabolized carbon in the solution before and after the separation of cells and of each precipitate represented the metabolized carbon of that particular fraction. Carbon dioxide, since it is the only appreciable volatile metabolite produced under the conditions employed (6, 20), was calculated from the differences in total carbon between parallel unfermented media and fermented cultures. Subsequent to the use of both alcohol and acetate in the fractionation, it was necessary to remove these compounds from samples taken for total carbon analyses. These samples were evaporated to dryness under reduced pressure. The residue was dissolved in a few ml.

TABLE I  
*Distribution of Metabolized Carbon in Cultures of Virulent and Attenuated Crown-Gall Bacteria*

Fraction No	Distribution of metabolized carbon	
	Virulent culture	Attenuated culture
	<i>per cent</i>	<i>per cent</i>
CO <sub>2</sub>	38	48
Cells	13	12
Gum (insoluble in 50% alcohol)	15	16
Polysaccharide (insoluble in 90% alcohol)..	18	13
Pb precipitate	6	18
Filtrate D	14	
Total	104	107

of distilled water, acidified with 1 or 2 drops of 1 N sulfuric acid, and the solution concentrated to almost dryness.

These determinations gave a rough idea of the distribution of the products in the fermentations. Typical metabolism balances are presented in Table I. The total percentages are somewhat high, probably because of errors in sampling and the assumption that all reducing substances reacting with the alkaline copper reagent in the sugar analyses contained the same percentage of carbon as glucose.

The data in Table I show no great differences in distribution of metabolized carbon in cultures of virulent and attenuated strains. The values for cells and gum and the total of the unidentified materials in the lead precipitates and Filtrate D are nearly the same in each case. However, in this and other experiments greater amounts of carbon dioxide and smaller amounts of the polysaccharide were produced by the attenuated organism.

*Isolation and Purification of Polysaccharide from Attenuated Cultures*—Isolation of the polysaccharide fraction was made by essentially the procedure used earlier (22) for the virulent culture, and already described above. Purification was accomplished by dissolving the polysaccharide in water and treating the solution with small successive portions of alcohol until it was opaque. It was then centrifuged and any barium remaining was removed by adding a few drops of 1 N sulfuric acid. Any excess of sulfate was then precipitated with barium carbonate, and the precipitate was removed by centrifugation. The clear supernatant liquid was then poured dropwise with stirring into 9 to 10 volumes of absolute ethyl alcohol. Repeatedly dissolving the polysaccharide in distilled water and reprecipitating it in alcohol resulted in increased purification. The final product, upon drying in a vacuum desiccator at 50°, was a nearly white, amorphous substance. After norit treatments of a solution of the substance in 50 per cent alcohol, the product was pure white. Three to four reprecipitations and one norit treatment usually sufficed for preparations from the virulent strain. However, the polysaccharide from the attenuated strain required five or six reprecipitations and three to four norit treatments before the brown color could be removed. The final product in each case dissolved readily in water and gave a clear, almost colorless, solution.

7 liters of medium containing sucrose as the carbon source were fermented by the attenuated strain and were treated by the above procedure with a yield of 14 gm. of purified polysaccharide. Two previous attempts at isolating this material from attenuated cultures yielded respectively 1.0 gm. from 2.2 liters and 0.84 gm. from 2.2 liters. Thus in three attempts an average yield of 0.94 gm. per liter was obtained.

*Properties of Polysaccharide from Attenuated Strain*—Determinations of the ash, specific rotations before and after hydrolysis, reducing sugar after hydrolysis, and nitrogen content were made. The ash content was 2.03 per cent, and the nitrogen content was about 0.15 per cent. On the ash-free basis, the specific rotation,  $[\alpha]_D^{20}$ , was  $-10.1^\circ$  ( $c = 2$ ,  $H_2O$ ). After hydrolysis specific rotation,  $[\alpha]_D^{20}$ , based on reducing sugar calculated as glucose, was  $+53.3^\circ$  ( $c = 2$ , 0.8 N HCl), thus indicating that the reducing sugar was glucose, a pure sample of which under the same conditions had a specific rotation of  $+52.6^\circ$ . Reducing sugar (glucose) accounted for 97 to 98 per cent of the substance. Hydrolysis in 1 N hydrochloric acid at 99° was complete in 90 to 105 minutes. These data are considered as good evidence that the polysaccharide from attenuated crown-gall bacteria is very similar, if not identical, with the polysaccharide from the virulent strain. A comparison of the properties of the polysaccharide from the attenuated strain with a number of polysaccharide preparations isolated from cultures of the virulent strain is given in Table II.

Additional evidence that polysaccharides of virulent and attenuated crown-gall bacteria are the same was obtained by Dr. R. E. Reeves<sup>1</sup> from specific rotations in cuprammonium hydroxide solutions. The method used is described by Reeves and Thompson (25). These polysaccharides show negative rotations in water and large positive rotations in cuprammonium solution (containing 15 gm. of copper and 240 gm. of ammonia per liter). Readings were taken on 0.5 per cent solutions at 25° with the Hg blue line (436 m $\mu$ ) and 0.5 dm. tubes. The specific rotations of the polysaccharide from virulent bacteria (Preparation 3, Table II) were -22° in water and +804° in cuprammonium. The corresponding specific rotations

TABLE II  
*Properties of Polysaccharide Preparations from Virulent and Attenuated Crown-Gall Bacteria*

Preparation No.	Substrate	Volume of culture	Yield per liter	Ash	$[\alpha]_D^{25}$ *	$[\alpha]_D^{25}$ after hydrolysis†	Reducing sugar*	Nitrogen
Virulent culture								
		ml.	gm.	per cent	degrees	degrees	per cent	per cent
1	Sucrose	4100	2.1	1.06	-10.5	+53.3	99-101	0.04
2	"	4000	3.5	0.35	-9.3	+55.1	99-102	0.08
3	"	3200	1.5	1.32	-9.9	+53.3	101-103	0.05
4	Fructose	1500	0.6	2.64	-8.9	+53.2	99-100	0.16
5	Glucose	1600	3.5	2.92	-10.3	+53.5	103-104	0.09
Attenuated culture								
6	Sucrose	7000	2.0	2.03	-10.1	+53.3	97-98	0.15

\* Calculated on the ash-free basis.

† The concentration was calculated from the reducing sugar (glucose) obtained.

for the polysaccharide from attenuated bacteria (Preparation 6, Table II) were -23° and +960°.

The preparations agreed well in negative rotation. The differences between +960 and +804 are beyond experimental error but are probably not significant when the differences in ash contents and purity of the two preparations are considered. As indicated in Table II, Preparation 3 from the virulent strain had a lower ash and a lower nitrogen content and a higher percentage of reducing sugar than the polysaccharide, Preparation 6, from the attenuated strain. The purer preparation would be expected to have a higher specific rotation.

The behavior of these preparations in cuprammonium is interpreted

<sup>1</sup>The authors wish to thank Dr. Reeves at the Southern Regional Research Laboratory, New Orleans, for these determinations.



by Dr. Reeves (23, 24) as indicating that the anhydroglucose units of the polysaccharide may be linked through the 1 and 2 positions.

*Polysaccharide Production by Virulent Crown-Gall Bacteria from Various Sugars*—The crown-gall bacterium utilizes a great variety of carbon and nitrogen sources (26, 29). It was desired to know whether the bacteria could convert other sugars as well as sucrose to the glucosan. Accordingly, appropriate media containing fructose, glucose, arabinose, xylose, and commercial sucrose, respectively, were fermented by the virulent strain, A6. The polysaccharide from each fermentation was isolated and was purified according to the procedures described above, and the properties of all preparations were compared.

A reasonable purity and a freedom from undue contamination for the various preparations reported in Table II were concluded on the basis of ash, nitrogen, total reducing sugar contents, and specific rotation. Ash contents ranged from about 0.4 to 3 per cent; those from the virulent strain cultured on sucrose were usually less than 1.5 per cent. Preparations from other sugars were higher. Nitrogen contents ranged from 0.04 to 0.15 per cent. Specific rotations were usually about  $-9^{\circ}$  to  $-10^{\circ}$  with small variations beyond these values.

Specific rotations both before and after hydrolysis of the polysaccharides which were formed from glucose and fructose, respectively, agreed well with those of the polysaccharide from sucrose. The maximum yields in 5 day fermentations from glucose and sucrose were about the same, *i.e.*, 3.5 gm. per liter. The average yield per liter of three preparations from sucrose was 2.4 gm. However, the preparation isolated from the fructose fermentation amounted to only 0.6 gm. per liter. The lower yield from fructose was explained partially by the amount of sugar fermented. In 5 days both virulent and attenuated organisms fermented 70 to 95 per cent of the sucrose. In fermentations to secure polysaccharide from glucose, fructose, and xylose the virulent organism had fermented 97, 59, and 58 per cent, respectively, of these sugars. However, it also seemed that fructose and other sugars not containing glucose units were either converted into glucose before the polysaccharide was formed or were degraded into smaller units, some of which were rebuilt into glucosan. In either case the over-all process was probably not as efficient as the seemingly more direct conversion of free glucose or glucose units (*e.g.*, in sucrose) into glucosan. Further evidence to this effect appeared when the yields of polysaccharide material from arabinose and xylose were considered.

The yield from arabinose was only 0.170 gm. from 1600 ml. of fermented medium. The specific rotation,  $[\alpha]_D^{20}$ , was  $-10.7^{\circ}$  ( $c = 0.9$ ,  $H_2O$ ) as calculated on the direct weight with no correction for ash content; specific rotation after hydrolysis was  $+49.7^{\circ}$  ( $c = 0.3$ ,  $H_2O$ ). The yield of poly-

saccharide material from a xylose substrate was 0.390 gm. from 1600 ml. of fermented medium. Specific rotation,  $[\alpha]_D^{20}$ , was  $-0.38^\circ$  (no correction for ash) before hydrolysis and  $+52.9^\circ$  ( $c = 0.2$ ,  $H_2O$ ) after hydrolysis. These preparations were obtained in such small quantities that adequate purification was difficult, and the expense of the pure substrates was too great to warrant carrying out fermentations on a scale large enough to assure that a few gm. of the polysaccharide material would be obtained. Preparations from all sugars were most satisfactorily purified when a yield of 5 gm. or more of the polysaccharide was obtained. The data on these two preparations were not sufficient to identify these polysaccharides with that produced from sucrose, glucose, or fructose, but the data obtained suggested that small amounts of the glucosan were also formed from arabinose or xylose.

*Attempts at Fermentation of the Polysaccharide*—Speculations as to the function of the polysaccharide in the cellular metabolism of the organism suggested that, if the polysaccharide were simply a reserve material, the organism should be able to utilize it as a source of carbohydrate. To test this possibility the following experiment was performed. Four series of 50 ml. flasks, each containing 10 ml. of the regular salts medium plus the carbohydrate under consideration at about 2 per cent concentration and 0.0813 mg. of manganous sulfate per 100 ml., were sterilized by autoclaving for 20 minutes at 15 pounds. They were inoculated with a 2.5 per cent 24 hour liquid culture of the virulent organism, and incubated at  $26^\circ$  ( $\pm 0.5$ ) with mechanical shaking during a period of several days. Since manganese is important in the metabolism of the organism (21), it was added in this experiment. The carbohydrate for each series was, respectively, (a) purified polysaccharide, (b) a mixture containing 1.69 per cent of polysaccharide plus 0.29 per cent of glucose, (c) glucose, and (d) sucrose.

The contents of one flask of each series were quantitatively transferred to a 250 ml. volumetric flask, diluted to volume, and samples taken for analysis of reducing sugar at the beginning and after 2, 4, 6, 10, and 14 days of fermentation. In the medium containing both glucose and polysaccharide, the reducing sugar value before hydrolysis was taken as the amount of glucose present and the difference between this value and that after hydrolysis in 1 N hydrochloric acid for  $1\frac{1}{2}$  to 2 hours at  $99^\circ$  represented the polysaccharide present. Utilization of sucrose was calculated (as glucose) from the reducing sugar present after hydrolysis with 1 N hydrochloric acid at  $70^\circ$  for 10 minutes. The data are summarized in Table III.

Analyses of cultures containing polysaccharide as the only source of carbohydrate showed that 0.8 to 10.1 per cent or an average of 5.9 per cent of polysaccharide disappeared in 14 days. In fermentations containing a mixture of polysaccharide and glucose all of the latter carbohydrate had

disappeared in 2 days and the total carbohydrate disappearing varied from 15 to 23 per cent with an average of 18.3. Since 15 per cent of the mixture was glucose, the polysaccharide utilized in this series was about 3 per cent. This indicated that, even after growth had been initiated on a readily utilizable substrate, the bacteria were still unable to metabolize the polysaccharide present. In contrast to the utilizations of glucose and of sucrose, 44.7 and 49.1 per cent, respectively, the polysaccharide was not utilized to any significant extent under the conditions employed.

The percentage utilizations of glucose and of sucrose are lower in this experiment than was generally observed. This was probably due to the limited aeration obtainable in 50 ml. flasks. Whereas ordinarily liter flasks were employed, it was not practical to use them in this experiment because of the limited amount of polysaccharide available.

TABLE III  
*Utilization of Polysaccharide by Virulent Crown-Gall Bacteria*

Length of incubation period	Source of carbohydrate							
	Polysaccharide		Glucose plus polysaccharide		Glucose		Sucrose	
	Present	Fermented	Present	Fermented	Present	Fermented	Present	Fermented
days	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
0	181.0	0	186.0*	0	183.8	0	189.6	0
2	174.4	5.3	151.0	18.5	133.0	27.6	126.6	33.2
4	163.4	9.7	151.0	18.5	120.5	34.4	114.8	39.5
6	162.8	10.1	142.5	23.3	115.5	37.2	123.5	34.9
10	179.6	0.8	156.5	15.8	113.0	38.5	106.2	44.0
14	174.7	3.5	157.3	15.3	101.6	44.7	96.6	49.1

\* This consisted of 158 mg. of polysaccharide and 28 mg. of glucose. Polysaccharide and sucrose were calculated (as glucose) from reducing sugar found after hydrolysis.

Further evidence on the availability of the polysaccharide as a nutrient was obtained in the following manner. A group of virulent cultures was grown by the usual methods for a period of several days. On the 5th day a group of flasks was removed, the contents combined, and the fermented medium examined for the polysaccharide fraction, as described above. The remaining flasks were allowed to incubate 14 days before they were likewise analyzed for polysaccharide. The yield from the 5 day cultures was 3.73 gm. per liter and from the 14 day cultures 5.71 gm. per liter. Sugar utilization in 5 days was 84 per cent and in 14 days was 100 per cent. The yield of polysaccharide from the 5 day fermentation was slightly higher than was obtained ordinarily; 3.5 gm. per liter were previously considered a good

yield. This represents a 17.5 per cent conversion of the sucrose to polysaccharide. The yield from the 14 day fermentation represents a conversion of 28.5 per cent of sucrose to polysaccharide. In order to account for this increased yield on the basis of the sucrose remaining after 5 days, it would be necessary to assume a conversion of 69 per cent of this sugar to polysaccharide. On the other hand the increase may be accounted for by assuming that an intermediate compound was converted into glucosan in the later stages of the fermentation. If the polysaccharide had been readily utilized, little would be found after 14 days, since under the conditions employed 99 to 100 per cent of the sucrose usually had disappeared in 6 days of fermentation.

The inability of the organism to metabolize the polysaccharide was further emphasized by the yield of this substance from very old cultures. Liquid cultures of the virulent organism were incubated for approximately 3 months. Aeration by mechanical shaking was provided during the first 5 days of the period and also for 3 days at the end of the incubation period. The polysaccharide was isolated, reprecipitated, dried, and weighed. From 1300 ml. of fermented medium a total yield of 6.5 gm. or 5 gm. per liter was obtained. Apparently the organism does not utilize the polysaccharide for energy purposes, and hence it must perform some function other than that of a reserve material.

#### DISCUSSION

That an attenuated strain of the crown-gall organism produced the same glucosan as a virulent strain adds another character in which these organisms are similar. Earlier workers (4, 28, 19, 27, 15) have made comparative studies of these strains but with one exception have found no differences which could be correlated with pathogenicity. Berge *et al.* (4) found giant colonies of attenuated crown-gall bacteria to have a greater ring pull with the du Noüy tensiometer than parallel colonies of a virulent strain, and attenuated liquid cultures also had a greater viscosity in the Saybolt viscosimeter than parallel virulent cultures. Since the viscosity of bacterial cultures appeared related to the production of gums, polysaccharides, and related substances (1), one might have expected to find larger amounts of polysaccharide in attenuated than in virulent cultures. The lower quantities of purified polysaccharide produced by the virulent cultures do not, however, exclude the possibility that other products may account for this difference in viscosity. It also remains for further study to show whether the variations in the amount of glucosan produced by virulent and attenuated strains have any relationship to their differences in pathogenicity.

Since the crown-gall organism was able to produce a glucosan from sugars which did not contain glucose units, this indicated a versatility not often

observed in polysaccharide production by microorganisms. Substrate requirements for polysaccharide formation mentioned in the literature are usually more specific. *Leuconostoc dextranicum* (3, 9, 10) and *Betabacterium vermiforme* (9) produced dextrans only from sucrose. *Leuconostoc mesenteroides* (31, 12) produced dextrans in quantity only from sucrose. Two strains of the latter organism produced slight amounts of polysaccharide when cultured on glucose (32).

The substrate requirements reported for the production of levans are also specific. *Bacillus mesentericus* and *Bacillus subtilis* formed levans only from sugars having terminal fructofuranose groups such as occur in sucrose or raffinose (11, 16). No polysaccharides were produced from melezitose, which consists of a fructofuranose group situated between two anhydroglucose units, nor from maltose, lactose, glucose, or fructose (fructopyranose). *Bacillus lactis* (5) produced a levan only from sucrose. A number of plant pathogens studied by Cooper and Preston (8) produced polysaccharides only when cultured upon media containing sucrose, and the polysaccharides were all of the levan type. These workers also pointed out that no evidence was yet available for the production of dextrans or pentosans by plant pathogens. The glucosan produced by the crown-gall organism now constitutes evidence for the formation of one of these compounds. These workers (8) included the crown-gall organism, *Phylomonas tumefaciens* (earlier designated as *Pseudomonas* and also *Bacterium tumefaciens*), among those plant pathogens consistently yielding no polysaccharide. Since they employed 3 volumes of alcohol for the separation of the polysaccharides studied, the glucosan produced by the crown-gall bacterium, and, which in our experience was precipitated only by 9 to 10 volumes of alcohol, doubtless was overlooked.

Reports of organisms which form the same polysaccharide from various substrates are not numerous. Polysaccharides from different species of rhizobia (17) varied in uronic anhydride contents, but the products formed by *Rhizobium meliloti* from sucrose and from mannitol were very similar in their contents of uronic anhydride and pentosan, and hence might be the same. The uronic anhydride contents of polysaccharides from *Rhizobium radicicolum* (7) grown on sucrose, glucose, or sorbitol varied with the substrate, being 18 per cent for sucrose, 4.4 per cent for glucose, and 3.6 per cent for sorbitol. The wide range in these figures suggests that the substance from sucrose is not identical with that from glucose or sorbitol, although the material from the latter two substrates might be the same. *Acetobacter xylinum* (2) formed from either glucose, fructose, galactose, sucrose, mannitol, or glycerol a cellulose which was identical with that contained in cotton.

That the crown-gall organism does not utilize the polysaccharide under

the conditions employed recalls the earlier work of Anderson (1) and Carruthers and Cooper (5). Anderson found that the purified gum isolated from rhizobia cultures, when incorporated as the source of carbohydrate into nutrient agar, did not support growth of the organism. Carruthers and Cooper showed that *Leuconostoc dextranicum* was unable to produce acid or reducing sugars from the dextran it synthesized, and no evidence of hydrolysis of the polysaccharide in culture was found.

#### SUMMARY

The isolation, purification, and properties of several polysaccharide preparations from cultures of virulent and attenuated strains, respectively, of the crown-gall bacterium have been described. More polysaccharide was obtained from virulent than from attenuated cultures. Specific rotations in aqueous and cuprammonium solutions and properties of hydrolysis products indicated that the polysaccharide from the attenuated strain was probably identical with the low molecular weight glucosan produced by the virulent strain.

Glucosan produced by the virulent strain from sucrose, glucose, and fructose varied in yield but possessed the same properties as judged by specific rotations, before and after hydrolysis, and reducing sugar content and hence were considered identical.

Very small quantities of polysaccharide material were also formed by the virulent organism from xylose and arabinose. Solubilities and specific rotations after hydrolysis suggested that these preparations were also of the glucosan type.

Under the conditions employed the glucosan was not readily utilized by the crown-gall bacterium as a source of carbohydrate. This was also indicated by the greater yields of the polysaccharide obtained from older cultures.

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# THE UTILIZATION OF *d*-AMINO ACIDS BY MAN

## II. CYSTINE\*

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In previous publications (1, 2) we have reported that the human metabolism of the *d* forms of tryptophane and phenylalanine differ from that of the *l* varieties, and that no differences could be detected in the utilization of the methionine enantiomorphs. Our attention was next directed to a study of the metabolism of *l*- and *dl*-cystine because of the practical implications arising from the probable presence of the unnatural form of cystine in acid hydrolysates of proteins (3) which are being proposed for parenteral feeding. In accordance with our adopted experimental technique, normal subjects were fed equal quantities of *dl*- and *l*-cystine and the metabolism of these compounds compared on the basis of the urinary output of cystine and other sulfur metabolites. Thus it was observed that the ingestion of *dl*-cystine caused a marked cystinuria in all subjects studied and that *l*-cystine did not. Indeed, the cystine output after the administration of *l*-cystine was found to be no greater than when no cystine was given. It was further noted that the feeding of racemic cystine induced an increase in the urinary output of total S, but failed to effect the excretion of methionine, indican, and inorganic S. These observations are interpreted to indicate that the unnatural isomer is only partially utilized by man.

### EXPERIMENTAL

Commercially available *l*(-)-cystine (Merck), which contained 11.64 per cent N by micro-Kjeldahl (4) and had a specific rotation of  $[\alpha]_D^{20} = -210^\circ$  in 1.01 *N* HCl (1 per cent), was used in these experiments. The requisite *dl*-cystine was prepared in this laboratory from 50 gm. of the optically active specimen described above by the du Vigneaud modification (5) of the Hoffman and Gortner procedure (3). The 25 gm. of cystine ob-

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tained by this process were found to contain 11.69 per cent N and to be optically inactive in 1.01 *N* HCl (1 per cent).

2 hours after the elimination of the night urine fasting subjects were given 2.4 gm. (0.01 *M*) of *l*(-)-cystine or *dl*-cystine, suspended in 240 cc. of water and an additional 120 cc. of water at the end of the 1st and also at the end

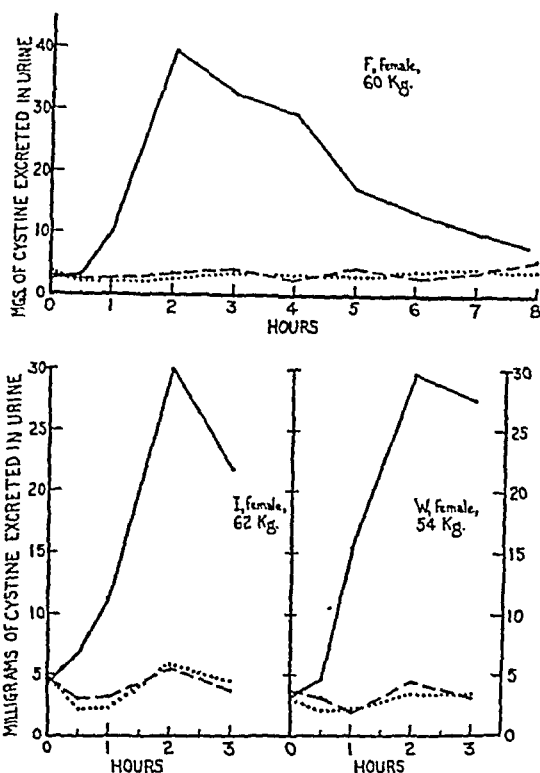


FIG. 1. Urinary output of cystine of fasting subjects after administration of 0.01 *M* (2.4 gm.) of *l*- and *dl*-cystine. The solid line indicates excretion after ingestion of the *dl*-form; the broken line after ingestion of the *l* variety; and the dotted line normal excretion.

of the 2nd hour in order to maintain uniform urine flow for the period of the experiment. The urine which was collected for the 2 hour period prior to administration of cystine is designated as "urine collected at 0 hours" in Table I. Urine was then collected at 0,  $\frac{1}{2}$ , 1, 2, and 3 hours and in some instances at 1 hour intervals thereafter to the 6th or 8th hour. The specimens were analyzed immediately for total N by micro-Kjeldahl (4), for

cystine by the Sullivan and Hess procedure (6), for methionine by the titrimetric method described by us (7), for total and inorganic sulfate sulfur by gravimetric techniques (8), and for indican by the Sharlit method (9).

From the representative data on the urinary cystine excretion shown in Fig. 1, it is observed that, whereas the ingestion of *l*-cystine does not result in a greater output of this amino acid than when no cystine is given, the

TABLE I

*Urinary Output of Nitrogen and Sulfur Compounds after Ingestion of 2.4 Gm. of l- and dl-Cystine by Normal Adult Human*

Subject A, male, 70 kilos.

Form fed	Time after ingestion	Urine volume	Total N	Total S	Inorganic S	Cystine	Methionine	Indican
	hrs.	cc.	mg.	mg.	mg.	mg.	mg.	mg.
<i>l</i> (-)-Cystine	0	280	521	36.9	25.2	6.4	62	1.52
	$\frac{1}{2}$	300	417	34.2	18.3	6.3	51	1.09
	1	200	326	36.8	17.7	4.2	23	0.82
	2	225	540	53.5	38.4	6.4	47	1.33
	3	170	640	66.5	42.0	5.7	38	1.39
	4	110	325	48.1	35.6	4.0	27	1.20
	5	105	320	44.5	34.0	4.0	24	1.18
	6	95	352	49.7	37.0	4.8	22	1.36
Total.....		1485	3441	370.2	248.2	41.8	294	9.89
<i>dl</i> -Cystine	0	325	484	45.6	12.7	7.7	41	1.63
	$\frac{1}{2}$	210	397	24.8	12.4	10.0	35	1.11
	1	170	280	27.3	17.4	19.4	24	0.75
	2	275	520	78.5	37.2	36.1	24	1.22
	3	120	364	62.7	39.2	34.8	33	1.28
	4	110	537	79.5	49.8	46.1	53	1.72
	5	100	498	66.9	41.6	32.6	32	1.54
	6	140	421	50.3	33.2	10.1	41	1.36
Total.....		1450	3501	435.6	243.5	196.8	283	10.61

feeding of the *dl* variety causes a marked cystinuria. These curves show a remarkable parallelism in all subjects.

Examination of the complete data (Table I) of a typical experiment further reveals that, although the urinary output of indican, methionine, and inorganic S for the 6 hour period following the ingestion of *l*- or *dl*-cystine fails to reflect any significant differences, the increased excretion of cystine and total S observed after the administration of the racemate points to the poor utilization of the *d* form. It is of particular interest to note that the feeding of racemic cystine resulted in the excretion of 155 mg. of cystine

and 65.4 mg. of total S in excess of that found after the feeding of an equal amount of *l*-cystine. Since the excess of total S is stoichiometrically equivalent to 247 mg. of cystine, it is obvious that the 92 mg. difference between this value and the excess cystine found (155 mg.) must represent sulfur (24.6 mg.) in a form other than cystine, methionine, indican, or inorganic sulfate sulfur. Our attempts to isolate and establish the chemical identity of this sulfur fraction have not been successful.

From the results of this and other experiments it appears that in 6 to 8 hours following the ingestion of 1.2 gm. of *d*-cystine, present in 2.4 gm. of the racemate, an excess of 120 to 180 mg. of cystine are excreted, which suggests the non-utilization of 10 to 15 per cent of the *d* isomer. The concomitant excess of total S found may represent a loss of an additional 10 per cent of the *d*-amino acid, but this cannot be definitely considered to be the case until the chemical nature of this S moiety has been established.

### Comment

It has been found that within 6 to 8 hours following the administration of 2.4 gm. of *dl*-cystine the characteristic urinary excretion pattern of the various S metabolites resulting from the presence of *d*-cystine shifts to that observed after an equal lapse of time of the ingestion of 2.4 gm. of *l*-cystine. Inasmuch as only 10 to 15 per cent of the *d*-cystine is recovered in the urine as excess in this interval, questions naturally arise as to the metabolic fate of the remaining 85 to 90 per cent of the *d*-cystine. Of this portion, it is possible that an additional 10 per cent may be lost as extra total S. Since the remainder of the *d*-cystine does not appear to yield an excess of inorganic sulfate S, methionine, or indican, it must be concluded that its metabolic fate in the human is similar to that of the *l* fraction. This would suggest, therefore, that unlike the result in the rat (4) some utilization of *d*-cystine occurs in man. The practical implication of this interpretation of our findings in human nutritional studies is, then, that some 25 per cent of the unnatural cystine which may be present in the acid digests of proteins now offered for parenteral use would escape utilization in the human. This loss, however, does not appear to be of such magnitude as to affect seriously the biological value of these preparations.

Our studies on the human metabolism of the *d*-amino acids to date have shown that *d*-tryptophane is utilized poorly or not at all, that *d*-cystine and *d*-phenylalanine are partially utilized, and that *d*-methionine is utilized as readily as the *l* form. A consideration of these observations leads us to believe that, except in instances of the formation of unphysiological products, *e.g.*, *d*-tryptophane, the utilization of the *d*-amino acids is one of degree rather than an all or none process. In other words, the availability of these substances is a function of two competing processes, (a) the

rate at which the organism can convert the unnatural into the natural variety and (b) the speed with which the respective *d*-amino acids are excreted by the kidney.

These investigations on the human utilization of the *d* forms of the amino acids are being continued and experiments with other amino acids will be reported in the near future.

#### SUMMARY

A comparison of the excretion data on the S metabolites following the administration of equal amounts of *l*- and *dl*-cystine suggests that some utilization of *d*-cystine occurs in the human.

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# ESTIMATION OF FORMALDEHYDE IN BIOLOGICAL MIXTURES

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The reaction of chromotropic acid with formaldehyde was discovered by Eegriwe (1), who studied it qualitatively. It was applied to visual photometry by Boyd and Logan (2), who found it to be sufficiently accurate for their purpose. Examination of their data indicates that transmittance was not related to concentration of formaldehyde according to Beer's law. In the present procedure,<sup>1</sup> extinctions follow Beer's law when measured in a Beckman quartz prism spectrophotometer, though not when measured in a Coleman clinical spectrophotometer. However, the data obtained by means of this instrument are reproducible with 1 per cent error and have been related by means of a chart to those obtained by means of the Beckman instrument.

The specificity of the chromotropic acid reaction with formaldehyde among organic substances, shown by Eegriwe (1), has been confirmed and extended: methanol, acetaldehyde, and formic acid do not react with chromotropic acid in the way formaldehyde does, nor do they interfere with the estimation of formaldehyde when present in the proportion of 10:1, or possibly more. The extinction curve, in the case of formaldehyde, shows maxima at 380, 480, and 570  $m\mu$ , the extinction at 480  $m\mu$  being about one-half that at 380 and 570  $m\mu$ ; whereas in the case of acetaldehyde there is a maximum at about 400  $m\mu$ , and methanol and formic acid do not react. Consequently, the wave-length chosen for estimation of formaldehyde was 570  $m\mu$ .

The present procedure consists essentially in mixing the solution containing formaldehyde with chromotropic acid in sulfuric acid and in heating

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<sup>1</sup> Eegriwe's chromotropic acid reaction has been adapted to spectroradiometric measurement by C. E. Bricker and H. R. Johnson in an investigation which was carried out simultaneously with the present work. Through their kindness, the manuscript was made available to the author, who finds that the details of the procedure are different, but that the essential spectroradiometric characteristics are the same. Their paper has been accepted for publication in *Industrial and Engineering Chemistry, Analytical Edition*.

the solution in a boiling water bath for 30 minutes or longer. The concentrations employed are 9 to 10 M sulfuric acid, 2 mg. of chromotropic acid per ml. of reaction solution, and formaldehyde in concentrations from 1.7 to 0.2  $\gamma$  per ml. of reaction solution.

Standardization of the chromotropic acid reaction can be accomplished conveniently by hydrolysis of known amounts of hexamethylenetetramine. 4 to 6 mg. of the tetramine are weighed, and transferred to 3 to 5 ml. of 2 N sulfuric acid for hydrolysis in a reaction tube like that designed by Hamilton and Van Slyke (3), or in a Thunberg tube. Hexamethylene-tetramine is easily purified by sublimation at pressures less than atmospheric and can be standardized by titration of ammonia evolved in acid hydrolysis according to the assay method of the United States Pharmacopoeia XII (4). The formaldehyde evolved by acid hydrolysis in an air-tight tube can be recovered quantitatively.

The conditions of the chromotropic acid are such as to cause hydrolysis of derivatives of formaldehyde that may be expected to be formed when biological material is treated with formaldehyde in the preparation of vaccines and toxoids. However, the chromotropic acid reaction may be applied to biological mixtures, before and after a reaction with Vorländer's reagent (5), 1,3-cyclohexanedione-5,5-dimethyl, and the estimate of free formaldehyde so obtained is reproducible with less than 2 per cent error.

The reaction of Vorländer's reagent with formaldehyde was studied, by means of the chromotropic acid reaction, with regard to pH and time of reaction. At pH 3.5, that of Vorländer's reagent in aqueous solution, the time required for complete reaction at 37° was 29 hours, but at pH 7 to 8 the time required was 30 minutes.

The chromotropic acid reaction and Vorländer's reaction can be combined with little or no change in procedure. Methylenebis(1,3-cyclohexanedione-5,5-dimethyl), the product formed from formaldehyde and Vorländer's reagent, is not hydrolyzed in 9 to 10 M sulfuric acid, and Vorländer's reagent in amounts employed does not react with formaldehyde under the conditions of the chromotropic acid reaction. Furthermore, when Vorländer's reaction is carried out at pH 7 to 8, at 37°, for 30 minutes, the reaction is complete, without causing removal of formaldehyde from its labile compounds, though prolonged association of Vorländer's reagent with so labile a derivative as that formed from arginine and formaldehyde does, in accordance with the observations of Wadsworth and Pangborn (6), cause a transfer of formaldehyde from arginine to that reagent, a process which may be called "*transmethylenation*." Yolk, allantoic fluid, chorioallantoic membrane, and embryo from developing chicken eggs do not contribute chromogenic material which will interfere with either the chromotropic acid alone or with the chromotropic acid reaction applied after

combination of free formaldehyde with Vorländer's reagent. The short time required for each analysis, 30 minutes, makes possible analysis of twenty samples in 4 hours, if the time required for preliminary preparation of the samples is counted.

The specificity of the chromotropic acid reaction with formaldehyde and the specificity of the ninhydrin reaction with  $\alpha$ -amino acids shown by Van Slyke, Dillon, MacFadyen, and Hamilton (7) make possible a specific method for determination of glycine. In agreement with Abderhalden (8), we have found that ninhydrin reacts with glycine to evolve formaldehyde. No other amino acid has been found to react with ninhydrin in this way. When the ninhydrin reaction is carried out at pH 1, the yield of formaldehyde measured by a 150 minute chromotropic acid reaction is a maximum and is 96 per cent of the amount which would represent complete conversion from glycine to formaldehyde.

#### *Apparatus—*

1. *Water bath.* The bath should be deep enough to insure that only the lips of the reaction tubes project above the cover. The cover is bored to permit insertion of the tubes into the bath, but it and the bath should be so designed as to prevent illumination of the solutions during the reaction. Uneven illumination causes irregularity in discoloration of the solutions.

2. *Glass reaction vessels.* The size of the vessel will depend on the reaction volume. The following vessels have been found to be satisfactory: for 50 ml., 200  $\times$  25 mm. tubes<sup>2</sup> marked to contain 50 ml.; for 25 ml., the Folin tubes<sup>2</sup> used for blood sugar analysis; for 10 ml., any test-tube which has been calibrated and marked to contain 10 ml.

3. *Spectroradiometer.*<sup>3</sup> We have used the Beckman quartz spectrophotometer and the Coleman clinical spectrophotometer,<sup>2</sup> model 6.

4. *Cuvettes.* Cuvettes of 1 cm. square cross-section were used in the Beckman instrument. Cylindrical cuvettes were used in the Coleman instrument. The cuvettes were standardized by selecting those of equal inner diameter and then by determining that position of the cuvette in the instrument that permitted reproducibility of transmittance to within 0.25 per cent. The inner diameter of a cylindrical cuvette, at a position opposite to the aperture for transmission of light from the diffraction grating to the cuvette, can be determined precisely enough from the relationship,  $d = \sqrt{4V \div \pi h}$ , where  $h$  is the height, in cm., between two marks made on the cuvette to correspond to the instrument aperture;  $V$  is the volume, in ml., between the two marks; and  $d$  is the inner diameter, in cm.

<sup>2</sup> Equipment listed in the Medical Supply Catalogue, Army Service Forces, United States Army.

<sup>3</sup> The term "spectroradiometry" is used in the sense proposed by Adams (9).



*Reagents—*

1. *Chromotropic acid (2,7-naphthalenedisulfonic acid-4,5-dihydroxy)*. We have used the practical grade manufactured by the Eastman Kodak Company and sold under the name 1,8-dihydroxynaphthalene-3,6-disulfonic acid, without further purification.

2. *Approximately 12.5 M sulfuric acid (sp. gr. 1.66)*. Prepared by mixing 2 volumes of concentrated sulfuric acid with 1 volume of water.

3. *Chromotropic acid-sulfuric acid reagent solution*. Prepared by dissolving 1 gm. of chromotropic acid in 100 ml. of water, filtering to remove insoluble sulfones, and adding 12.5 M sulfuric acid in the amount needed to make a total volume of 500 ml. The reagent solution is kept in stoppered bottles, protected from illumination. When freshly prepared, the reagent shows a per cent transmittance greater than 91 at a wave-length of 570  $\mu$ . The concentration of sulfuric acid in the reagent solution should be 9.5 to 10 M, as determined by dilution and subsequent acidimetric titration or by specific gravity measurements (1.50 to 1.53). *The most important consideration of sulfuric acid concentration is that it should not be less than 9 M when the reagent solution and the solution to be tested for HCHO are mixed and ready for the chromotropic acid reaction. The reagent is unusable when the per cent transmittance becomes reduced to 75, as it will on standing for a week or longer.*

4. *Dimethyldihydroresorcinol (1,3-cyclohexanedione-5,5-dimethyl)*. We have used the product manufactured by the Eastman Kodak Company. A solution containing 0.2 gm. in 100 ml. of water, or of buffer solution at pH 7 to 8, is prepared anew at intervals of no longer than 1 week; as observed by Vorländer (5), solutions of this substance cause hydrolysis with evolution of HCHO, which then combines with unhydrolyzed reagent.

5. *Buffer solution at pH 7 to 8*. Used for diluting either the material to be tested for HCHO or for preparing a solution of dimethyldihydroresorcinol; in either case, in order that Vorländer's reaction be carried out at pH 7 to 8. We have used the citric acid-phosphate buffer mixtures of McIlvaine (10).

*Procedure for Chromotropic Acid Reaction*

A known volume of aqueous solution containing formaldehyde, in an amount which will not exceed 1.7  $\gamma$  per ml. of HCHO-chromotropic-sulfuric acid reaction solution, is delivered into a reaction tube. Then enough chromotropic-sulfuric acid reagent solution is added to make a known reaction volume. The solution is mixed by means of a thin, footed stirring rod. As a control, an equal volume of a solution containing all reagents except formaldehyde is tested in the same way. Restrictions with regard to volume of the sample, concentration of formaldehyde in the sample, and

volume of HCHO-chromotropic-sulfuric acid reaction solution are indicated by the formula,  $(S \times C)/R \leq 1.7$ , where  $S$  is the volume of sample to be tested, in ml.;  $C$  is the concentration of HCHO in the sample, in terms of micrograms per ml.; and  $R$  is the volume, in ml., of the HCHO-chromotropic-sulfuric acid reaction solution. The volume of aqueous solution taken for analysis,  $S$ , must not exceed 0.1 of the volume of the reaction solution prepared from 10 M sulfuric acid reagent solution,  $R$ , lest the concentration of sulfuric and chromotropic acids be diminished to a degree causing prolongation of the minimum reaction time.

The reaction solutions and control solution are lowered into a water bath which has been brought to a vigorous boil. Strips of gauze or other material are interposed between the tubes and the openings in the cover, to prevent steam from leaving the bath near the mouths of the tubes. The tubes are not stoppered. Consequently, the foregoing precaution serves to prevent drip of condensed steam into the reaction solutions. Boiling is continued for 30 minutes or longer, and the level of the reaction solution should be lower than the level of water in the bath throughout the boiling period.

When the reaction has been completed, the tubes are removed from the boiling water bath and cooled to room temperature. Under the present conditions, the volume of reaction solution, which increases during the reaction, requires very little adjustment after the solution is cooled to room temperature; the decrease in volume caused by evaporation of water is less than 2 per cent of the volume of the reaction solution. However, if the concentration of sulfuric acid in the reaction solution exceeds 12 M, water will be absorbed from the air faster than it evaporates from the reaction solution, particularly on humid days.

Spectroradiometric measurement is made at a wave-length of 570  $\mu$ . Temperature variations between 22–30° do not affect the extinctions significantly. If the reaction solutions are stoppered and kept in the dark, no change will occur in the extinction due to specific absorbing substance for at least 3 days, though both the reaction solution and control solution will become discolored. If the reaction solution is turbid, as in analyses of some biological materials, it may be cleared by filtration through sintered glass. When the Coleman instrument is employed, the zero adjustment (adjustment of the galvanometer scale to zero when the barrier layer cell receives no light) must be made carefully before and, if necessary, during a series of measurements. The control solution is used to set the galvanometer scale to 100 per cent transmittance.

In the event that the concentration of formaldehyde in the test material is unknown, a sample is analyzed in the way described, but if the resulting extinction is too great for spectroradiometric measurement the reaction

solution and control solution are diluted with 9 to 10 M sulfuric acid until the extinction becomes measurable. The extent of dilution with acid so determined is the extent of dilution of the aqueous test material with water that will be required prior to precise estimation of the formaldehyde content.

When cuvettes of 1 cm. square cross-section are used in the Beckman instrument, the concentration of formaldehyde in the reaction solution can be computed from the equation,  $E_{570} = 0.57C'$ , where  $E_{570}$  is the extinction observed at a wave-length of 570 m $\mu$  and has been corrected for the extinction due to the control solution;  $C'$  is the concentration of HCHO reacting with chromotropic acid, in terms of micrograms per ml. of reaction solution; and 0.57 is the extinction coefficient.

When cylindrical cuvettes are used in the Coleman instrument, the concentration of formaldehyde,  $C'$ , can be calculated from the relationship of  $(E_{570} \div d)$  in the Coleman instrument to  $E_{570}$  in the Beckman instrument, shown in Fig. 6, where  $E_{570}$  has the significance indicated previously and  $d$  is the inner diameter of the cuvette, in cm.

#### *Procedure for Vorländer's Reaction*

2 ml. of solution containing formaldehyde, in amount not exceeding 150  $\gamma$ , are mixed with 2 ml. of dimethyldihydroresorcinol solution. The solution is immediately warmed to 37° and is held at that temperature for 30 minutes.

For completion of this reaction in 30 minutes, the solution must be buffered at pH 7 to 8. The amounts of formaldehyde used in the preparation of vaccines are usually such as to require dilution prior to the chromotropic acid reaction. In these cases, and in similar cases in which the amount of HCHO to be tested is known approximately, the material to be tested may be diluted with McIlvaine's buffer (0.2 M  $\text{Na}_2\text{HPO}_4$ , 0.1 M citric acid), or presumably with other buffers, at the desired pH. Otherwise it is necessary to prepare Vorländer's reagent in buffer instead of water. This alternative raises a point of caution. Increased solubility at pH 7.0 makes possible a solution containing 1 gm. of dimethyldihydroresorcinol per 100 ml., as compared with a saturated aqueous solution (pH 3.5) containing 0.45 gm. per 100 ml. (6). The concentration of Vorländer's reagent in solution with HCHO should not, however, exceed 0.2 per cent, or, twice that directed in the present procedure, because greater concentrations accelerate transmethylenation and also reduce the extinction due to the reaction of formaldehyde with chromotropic acid.

After 30 minutes at 37°, 2 ml. aliquots are taken for the chromotropic acid reaction, without waiting for the Vorländer's reaction solutions to cool to room temperature, and mixed with the chromotropic-sulfuric acid reagent

immediately. Vorländer's reaction is stopped in 9 to 10 M sulfuric acid. There will be many instances not demanding the careful attention to time directed in this procedure, but unless transmethylenation can be shown not to occur for a particular case the directions should be followed to avoid misleading interpretation of the results.

### *Procedure for Biological Mixtures*

The present procedure was designed for ease of computation of the results. If the formaldehyde determinable by direct chromotropic acid reaction is denoted as  $A$ , and that determinable after the Vorländer reaction as  $B$ , by the present procedure  $A$  and  $B$  will be comparable with regard to dilution. The difference,  $A - B$ , can be taken directly to represent free formaldehyde.

The concentration of free  $\text{HCHO}$  in the test material can then be computed from the equation,  $(F) = (A - B)/0.985D$ , where  $(F)$  is the concentration of free formaldehyde in the test material;  $A$  and  $B$  have the significance attributed to them in the previous paragraph;  $D$  is the extent of dilution of the test material prior to analysis  $A$ ; and 0.985 is a factor to correct for the fact that in the present procedure, with freshly prepared solutions of Vorländer's reagent, whether the reaction time is 30 minutes or longer, Vorländer's reagent reacts with 98.5 per cent of formaldehyde. When aqueous solutions of dimethyldihydroresorcinol are stored at room temperature, they will react with  $\text{HCHO}$  to yield a lower numerical value than 0.985 for the ratio,  $(A - B)/A$ . We have not found that the factor changes in 1 week's time of storage, but in 3 months the factor was found to be 0.96.

The concentration of formaldehyde that has reacted with biological material, designated as  $(R)$ , can be computed from the equation,  $(F_0) - (F) = (R)$ , where  $(F_0)$  is the concentration of formaldehyde in the biological mixture at the time that the formaldehyde and biological mixture were mixed together, and  $(F)$  has been determined by analyses, as previously described. It should be noted that  $(R)$  need not represent compounds of formaldehyde with other substances, but instead may be formic acid or methanol.

### EXPERIMENTAL

#### *Chromotropic Acid Reaction*

*Concentration of Sulfuric Acid*—The effect of varied concentration of sulfuric acid on the rate of reaction of chromotropic acid with formaldehyde is shown in Fig. 1. The reaction in 3 M acid is so slow as to be scarcely noticeable in 30 minutes, but in 9 M or stronger acid it is at an end in 30 minutes. The results shown in Fig. 1 were obtained with a concentration of 2 mg. of chromotropic acid per ml. of reaction solution.

Another effect of varied acidity has to do with blank analyses. Chromotropic acid in sulfuric acid becomes discolored when heated, the more rapidly the stronger the acid: the per cent transmittance at  $570\text{ m}\mu$ , after heating the reagent solution for 30 minutes in a boiling water bath, is about 91 in 9 to 10  $\text{M}$  acid, is not much changed in 11  $\text{M}$  acid (88), but is 78 or less in 12  $\text{M}$  acid. Consequently the analytical results are more variable and the amount of  $\text{HCHO}$  that can be measured accurately is reduced when the reaction is carried out in sulfuric acid of concentration greater than 11  $\text{M}$ .

Acidity also affects the numerical value of the extinction coefficient. In concentrations of sulfuric acid greater than 8.5  $\text{M}$  the extinction coefficient

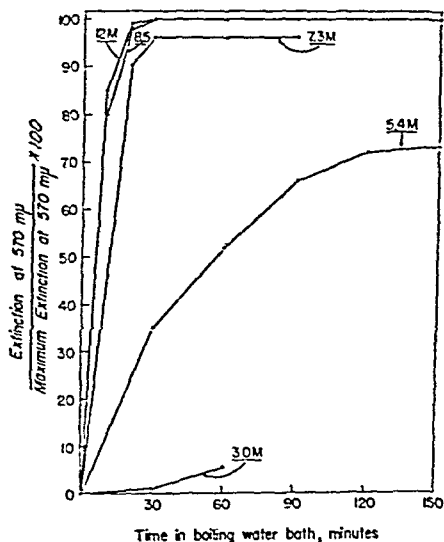


FIG. 1. Relationship between extinction at  $570\text{ m}\mu$  and the concentration of sulfuric acid.

is constant, but as the concentration is lessened (as by dilution with water) the coefficient is at first increased and then decreased. As a result, dilution of chromotropic-sulfuric acid- $\text{HCHO}$  reaction solutions by means of water can yield different extinctions than dilution by means of sulfuric acid. Extinction is proportional to the concentration of reacting  $\text{HCHO}$  in the case of dilution at constant acidity, but differences as great as 10 to 15 per cent can occur in the case of dilution by means of water.

**Concentration of Chromotropic Acid**—The effect of varied concentration of chromotropic acid is shown in Fig. 2. The concentration must be 1.5 mg. or more per ml. of reaction solution to make possible complete reaction with 1.7  $\gamma$  of  $\text{HCHO}$  in 30 minutes.

*Spectroradiometric Characteristics*—The engineering characteristics of the spectroradiometers and cuvettes employed in the present experiments are shown in Table I.

The absorption curves obtained by the reaction of formaldehyde with chromotropic acid are shown in Fig. 3, for the Beckman instrument, and in Fig. 5 for the Coleman instrument. When the Beckman instrument is used, the extinctions at varied wave-lengths are proportional to the concentration of HCHO reacting with chromotropic acid. It will be noticed that the minor peak at  $480\text{ m}\mu$  is somewhat obscured when the Coleman instrument

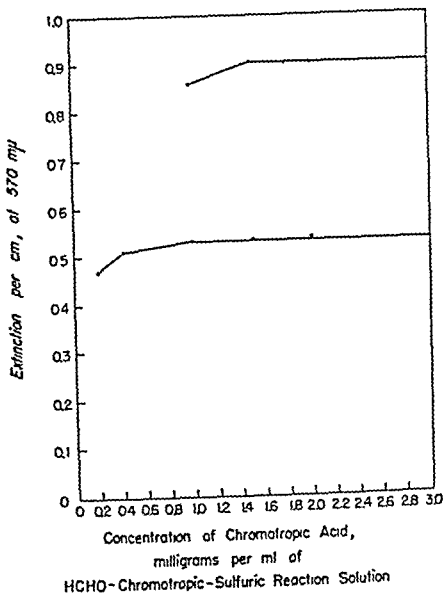


FIG. 2. Relationship between extinction at  $570\text{ m}\mu$  and the concentration of chromotropic acid. Upper line,  $1.6\text{ }\gamma$  of HCHO per ml. of reaction solution; lower line,  $0.94\text{ }\gamma$  of HCHO per ml. of reaction solution.

is used, presumably because of differences in engineering characteristics shown in Table I.

The absorption curves obtained by the reaction of substances allied to formaldehyde are shown in Fig. 4, which also includes the curve for the reaction of ninhydrin (indanetrione hydrate) with chromotropic acid reagent. Comparison of Figs. 3 and 4 reveals the specificity of the HCHO-chromotropic-sulfuric acid absorption curve. The curve in the case of the furfural is complicated by oxidation of furfural independently of the chromotropic acid reaction. When these substances, including furfural,

are mixed with formaldehyde in the proportion of 10:1 or less, they do not interfere with the quantitative estimation of formaldehyde, as shown in Fig. 5.

The specificity of the chromotropic acid reaction with formaldehyde is shown in Table II, in which most of the substances tested and found not to react with chromotropic acid are compiled from Eegriwe's paper (1).

The determination of the extinction coefficient,  $K'$ , at a wave-length of

TABLE I  
*Characteristics of Spectroradiometers and Cuvettes Used in Present Work*

Group designation, cuvettes	Manufacturer and catalogue No.	Inner diameter	Wall thickness	No. of cuvette
		cm.	cm.	
A	Medical Department Supply Catalogue, Army Service Forces, United States Army, 1942; Item 17506	1.400	0.061	3
		1.400	0.062	4
		1.397	0.060	5
		1.400	0.058	8
		1.386	0.064	10
B	Coleman Electric Company, Inc.; No. 6-304	1.638	0.107	1
		1.625	0.114	2
		1.631	0.105	8
		1.601	0.112	9
C	Hellige Incorporated; No. 452-AB	1.773	0.117	1
		1.760	0.106	2
		1.765	0.112	3
		1.769	0.112	4
		1.773	0.117	5
D	National Technical Laboratories; Corex	1.001*	0.140	831*
		0.999	0.140	804
		1.000	0.144	828
		1.001	0.145	834

Spectroradiometer	Resolution of light	Slit width	Nature of responding cell
		mμ	
Beckman	Quartz prism	1-2	Special C-7032
Coleman, clinical	Diffraction grating	30-35	Barrier layer

\* Manufacturer's figures.

570 mμ was made in several ways. The following method was finally adopted as being most convenient and precise. A weighed quantity of pure hexamethylenetetramine, 4 to 6 mg.,<sup>4</sup> in a small tube, was placed in a Hamilton-Van Slyke reaction tube ((3) Fig. 1, A). Then 3 to 5 ml. of 2 N sulfuric acid were placed around the small tube, air was removed by suction,

<sup>4</sup> Increasing the amount to 50 mg. or more in 5 ml. of 2 N sulfuric acid delays hydrolysis, so that in 2 hours the extent of hydrolysis is 85, 81, and 77 per cent for 50, 100, and 200 mg. of hexamethylenetetramine, respectively.

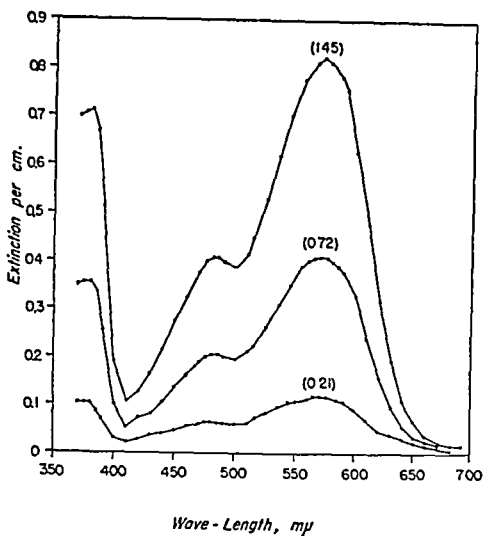


FIG. 3. Absorption curves for the reaction of formaldehyde with chromotropic acid, when the Beckman instrument was used. Numbers in parentheses refer to the concentration of formaldehyde, in terms of micrograms per ml. of HCHO-chromotropic-sulfuric acid reaction solution.

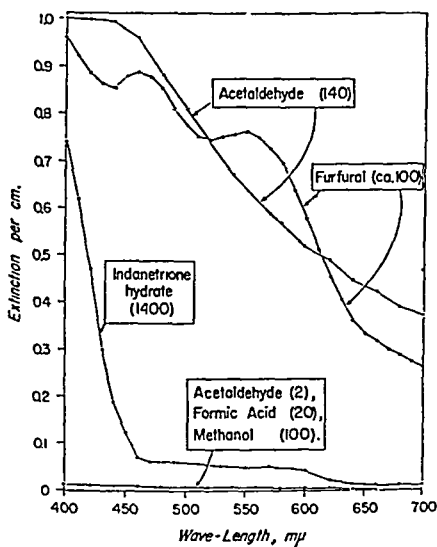


FIG. 4. Absorption curves for the reaction of chromotropic acid with substances allied to formaldehyde, when the Coleman instrument was used. Numbers in parentheses refer to the concentrations of substances tested, in terms of micrograms per ml. of substance-chromotropic-sulfuric acid reaction solution.



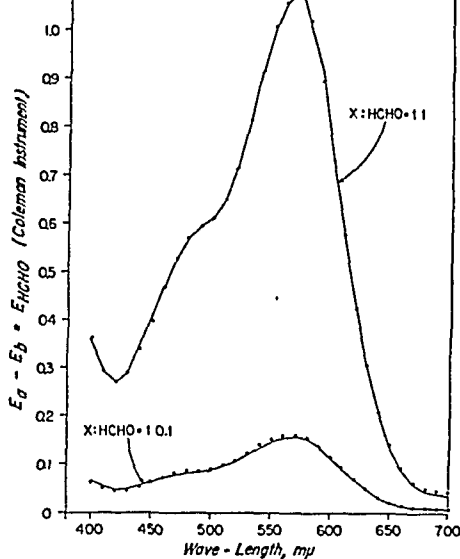


FIG. 5. Quantitative estimation of formaldehyde in the presence of allied substances.  $X$  is methanol, formic acid, acetaldehyde, or furfural tested separately.  $E_a$  is the extinction due to  $X + \text{HCHO}$ ;  $E_b$  is the extinction due to  $X$  alone.  $E_{\text{HCHO}}$  is the extinction calculated to be due to formaldehyde. Continuous lines are absorption curves for formaldehyde in the absence of  $X$ . Dots are absorption curves calculated from  $E_a - E_b$ .

TABLE II  
Substances Tested and Found Not to React with Chromotropic Acid

Carbonyl compounds		Miscellaneous
Aldehydes	Ketones	Alcohols
Acetal*	Acetone	Methanol*
Propional	Dimethyldihydroresorcinol*	Ethanol
Butyral	Methylenebis(1,3-cyclohexanedione-5,5-dimethyl)*	Glycerol
Isobutyral		Biological materials
Isovaleral		Chicken egg yolk
Glyceral	Acids	Embryo
Oenanthal	Formic*	Allantoic fluid
Crotonal	Levulinic	Chorioallantoic membrane*
Chloral hydrate	Glycolic	<i>Shigella sonnei</i> *
Glyoxal	Oxalic	Amino acids
Benzal	Gallic	Glycine*
Oxybenzal	Acetic	Arginine*
Vanillin	Sugars	
Salical	Glucose	
Phthalal	Fructose	
Isophthalal	Mannose	
Terephthalal	Lactose	
Cinnamal	Galactose	

\* Tested quantitatively in the present work. Most of the observations were made by Eegriwe (1).

and the larger tube was made air-tight by a turn of its stopper. The sample was mixed with the acid by several inversions of the reaction tube, and then immersed in a boiling water bath for 2 hours to accomplish complete hydrolysis to ammonia and formaldehyde. At the end of the boiling period the solution was cooled to room temperature and quantitatively transferred<sup>5</sup> to a volumetric flask, and diluted with water to known volume. 1 ml. aliquots of the diluted solution, called hydrolysate in Table III, were tested by the chromotropic acid procedure. The results obtained at varied times of hydrolysis are shown in Table III, which also includes results obtained from hydrolysis of paraformaldehyde. The computation of the extinction coefficient was made according to the general equation,  $K' = E/C'L$ , where  $K'$  is the extinction coefficient;  $E$  is the observed extinction at 570 m $\mu$ ;  $C'$  is the concentration of formaldehyde calculated to be reacting, in terms of micrograms per ml. of reaction solution; and  $L$  is the length of the path of radiant energy through the cuvette, in cm. The prime on  $K$  and  $C$  signifies that they do not refer to the concentration of specific substance absorbing radiant energy, which in this case is unknown. In turn,  $C'$  is equal to  $(W \times f)/(V_h \times V_r)$ , where  $W$  is the weight of substance analyzed, in micrograms;  $f$  is the factor relating gm. of substance to gm. of formaldehyde evolved from it, and is equal to 1 and 1.285, in the cases of paraformaldehyde and hexamethylenetetramine, respectively; conversely,  $f$  is 0.1027 in calculation of the amount of formaldehyde equivalent to a given weight of methylenebis(1,3-cyclohexanedione-5,5-dimethyl);  $V_h$  is the volume to which the sample  $W$  is diluted prior to the chromotropic acid reaction; and  $V_r$  is the volume of chromotropic acid reaction solution in which 1 ml. of the volume  $V_h$  is analyzed, both volumes being given in terms of ml.

Table III shows that the extinction coefficient, determined according to the procedure described in the foregoing paragraph, and by means of the Beckman instrument, varied from 0.563 to 0.577 at a wave-length of 570 m $\mu$ . The average extinction coefficient was 0.568 in the case of paraformaldehyde and of hexamethylenetetramine. The paraformaldehyde was not tested for purity. Hexamethylenetetramine was purified by three resublimations under reduced pressure, less than 40 mm. of Hg, and at temperatures from 100-200°. It was assayed according to the United States Pharmacopoeia XII (4); the ammonia evolved by hydrolysis in sulfuric acid and after distilling off HCHO for 3 hours was determined by acidimetric titration

<sup>5</sup> When the tube and solution have cooled, the pressure in the tube is less than atmospheric. Nevertheless, without first admitting water through the side arm to restore atmospheric pressure (a procedure which, almost filling the tube with liquid, makes transfer difficult), opening the vessel to air causes no loss of formaldehyde by vaporization.

to be 100.5 per cent of that amount expected from complete hydrolysis of pure hexamethylenetetramine.

Oxidation of Merck's neutral reagent formaldehyde by iodine, according to the directions of Cumming, Hopper, and Wheeler (11), with 20 mg. samples of HCHO, gave results which in conjunction with simultaneous chromotropic acid analyses were equivalent to an extinction coefficient of  $0.573 \pm 0.004$ . The same solution of formaldehyde, when analyzed gravi-

TABLE III

*Determination of Extinction Coefficient at 570  $\mu$ , by Means of Hydrolysis of Paraformaldehyde and Hexamethylenetetramine*

	Conditions of hydrolysis			Volume of chromotropic reaction solution containing 1 ml. of (H)	Concentration of HCHO in chromotropic reaction solution (C')	Extinction, observed at 570 $\mu$ (E)	Extinction coefficient (K' = E/C'L)
	Weight of sample (W)	Time of hydrolysis* (min.)	Volume of hydrolysate† (H)				
	mg.	min.	ml.	ml.	$\gamma$ per ml.		
Paraformaldehyde, $C' = 1000W/RH$	5.887	30	100	50	1.177	0.649	0.551
	4.937	30	100	50	0.987	0.539	0.546
	5.257	60	100	50	1.051	0.597	0.568
	4.827	60	100	50	0.965	0.557	0.577
	4.127	60	100	50	0.825	0.470	0.570
	5.625	60	100	50	1.125	0.642	0.571
	5.750	60	100	50	1.150	0.648	0.563
	6.230	180	100	50	1.246	0.699	0.561
	6.862	180	100	50	1.372	0.772	0.563
	6.924	30	250	50	0.712	0.392	0.551
Hexamethylenetetramine, $C' = 1285W/RH$	7.459	30	250	50	0.767	0.429	0.560
	7.607	60	250	50	0.782	0.429	0.548
	0.500	60	50	10	1.285	0.730	0.568
	0.500	60	50	10	1.285	0.720	0.563
	6.640	120	250	50	0.683	0.384	0.563
	6.850	120	250	50	0.704	0.401	0.569
	5.091	180	250	50	0.523	0.297	0.568

\* In 3 to 5 ml. of 2 N sulfuric acid.

† After dilution with water.

‡  $L = 1$  cm. in the cuvettes used.

metrically by means of Vorländer's reaction, gave results equivalent to an extinction coefficient of  $0.576 \pm 0.006$ . However, the extinction coefficient calculated from the results of precipitation of HCHO as methylenebis-(1,3-cyclohexanedione-5,5-dimethyl) was corrected for incompleteness of the Vorländer reaction under the present conditions; the weight found was multiplied by 1.02, to correct for a reaction only 98 per cent complete. The solubility of the bis compound under the present conditions of precipitation was not determined. Application of a correction for solubility

in the calculation of the extinction coefficient would reduce the value from 0.576 to some lower value. Since the gravimetric determination of formaldehyde by means of Vorländer's reaction, as usually carried out, does not include testing the filtrate for  $\text{HCHO}$ , the present method is described as follows:

20 ml. of solution containing 40 mg. of  $\text{HCHO}$  were mixed with 500 ml. of solution containing 2 gm. of dimethyldihydroresorcinol in 0.1 M  $\text{KH}_2\text{PO}_4$ . The pH was adjusted from 4.1 to 4.7 by adding concentrated  $\text{NaOH}$  drop by drop. A control solution, in which water was substituted for formaldehyde, was prepared in the same way. Both solutions were placed in an oven at  $37^\circ$  for 18 hours; the control solution showed no precipitate. The precipitate was washed with 0.001 M  $\text{KH}_2\text{PO}_4$  and dried over sulfuric acid in an evacuated desiccator for 3 days to constant weight.<sup>6</sup> The completeness of Vorländer's reaction was tested by chromotropic acid analysis of the filtrate from the precipitation mixture, and by analysis of the solution containing only Vorländer's reagent. No  $\text{HCHO}$  was detectable in the control solution; the amount found in the filtrate was equivalent to 2 per cent of that submitted to the Vorländer reaction. Samples of the bis compound were tested in the chromotropic acid reaction; 10 mg. yielded no  $\text{HCHO}$ ; 20 mg. yielded an amount not greater than 0.7  $\gamma$  of  $\text{HCHO}$ , or 0.004 per cent of an amount expected if the bis compound was completely hydrolyzed to evolve  $\text{HCHO}$ . The correction for incompleteness of Vorländer's reaction would seem to be valid, since the bis compound is not hydrolyzed and Vorländer's reagent does not yield formaldehyde under the experimental conditions tried.

The determination of the extinction coefficient by three different kinds of analytical procedures yielded numerical values in good agreement: Hydrolysis of hexamethylenetetramine gave  $0.567 \pm 0.004$ ; iodine oxidation of a solution apparently free from paraformaldehyde gave  $0.573 \pm 0.004$ ; and gravimetric estimation by reaction with dimethyldihydroresorcinol gave  $0.576 \pm 0.006$ , for triplicate analyses. On the basis of these results, the extinction coefficient is taken to be 0.57, and this value is employed in all subsequent calculations in this paper.

The proportionality of extinction to concentration of formaldehyde is shown in Fig. 6, for measurements made in the Beckman instrument. Proportionality did not obtain when the Coleman instrument was used, but the results are reproducible with less than 1 per cent error.

The proportionality of extinction to the inner diameter of the cylindrical cuvettes used in the Coleman instrument is shown in Table IV, in com-

<sup>6</sup> Drying in an oven at  $110^\circ$ , as directed by Wadsworth and Pangborn (6), was also tried. In 2 hours, the time advocated, there was no apparent loss by decomposition (the extinction coefficients were the same as in the case of drying at room temperature). However, longer periods of drying caused loss of weight equivalent to 2 per cent per 2 hour period, and in 2 days charring was obvious. Decomposition is not noticeable during the melting point determination; the melting point was  $191^\circ$ , and in good agreement with previously published figures,  $184$ – $192^\circ$ .

parison with extinctions obtained by using cuvettes of 1 cm. square cross-section in the Beckman instrument. Cuvettes B, supplied with the Coleman instrument, gave results which were consistently about 2 per cent lower than those associated with the use of the other cuvettes, the difference being due presumably to differences in optical characteristics of the glass.

The volume of the reaction solution was varied from 10 to 50 ml. without affecting the proportionality of extinction to concentration of HCHO, when either instrument was employed. Coleman instruments in three different laboratories, each calibrated by means of a didymium filter, yielded results agreeing with less than 2 per cent error.

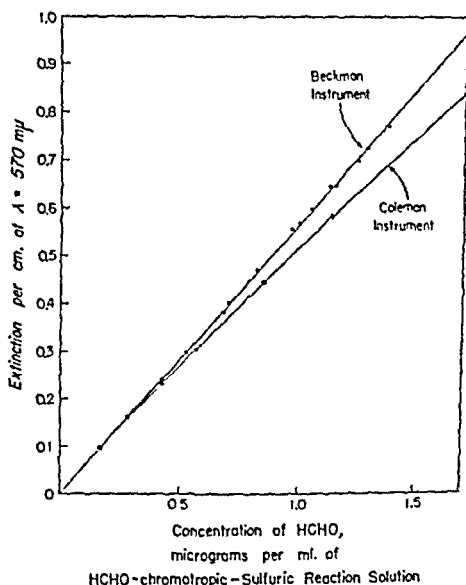


FIG. 6. Relationship between extinction at 570  $m\mu$  and the concentration of formaldehyde.

The results indicate that the chromotropic acid method is generally applicable to spectroradiometric instruments. For standardization of the reaction, preparation of calibration curves, determination of extinction coefficient, hydrolysis of hexamethylenetetramine is preferred.

#### *Reaction of Formaldehyde with Dimethyldihydroresorcinol*

*Rates of Reaction at Varied pH*—Study of the rates of reaction of formaldehyde with dimethyldihydroresorcinol (1,3-cyclohexanedione-5,5-dimethyl), Vorländer's reagent, has usually been limited to gravimetric

estimations, and the pH has been variously considered. Vorländer (5) advocated adjusting the precipitation mixture to a pH corresponding to the end-point of methyl orange (pH 4.4), and showed that the formation of methylenebis(1,3-cyclohexanedione-5,5-dimethyl), also called formaldi-

TABLE IV

*Proportionality of Extinction at 570 Mμ to Inner Diameter of Cylindrical Cuvettes*

Measurements in Coleman instrument									Beckman instrument; extinction at 570 mμ; L = 1 cm.
Cuvettes A			Cuvettes B			Cuvettes C			
Cuvette No.*	Extinction at 570 mμ (E')	E'/d†	Cuvette No.*	Extinction at 570 mμ (E')	E'/d†	Cuvette No.*	Extinction at 570 mμ (E')	E'/d†	
3	1.011	0.722	1	1.155	0.705	1	1.270	0.716	0.814
4	1.011	0.722	2	1.155	0.710	3	1.270	0.720	0.812
8	1.011	0.722	8	1.155	0.708	4	1.270	0.718	0.816
10	1.000	0.721			0.708	5	1.260	0.717	
Mean.....		0.722			0.708			0.718	0.814
3	0.745	0.532	1	0.854	0.521	1	0.930	0.525	0.583
4	0.745	0.532	2	0.854	0.525	3	0.930	0.527	0.582
8	0.745	0.532	8	0.854	0.524	4	0.930	0.526	0.583
10	0.745	0.537				5	0.925	0.526	
Mean.....		0.533			0.523			0.526	0.583
3	0.459	0.328	1	0.527	0.322	1	0.585	0.330	0.351
4	0.465	0.332	2	0.527	0.324	3	0.585	0.331	0.351
8	0.465	0.332	8	0.527	0.323	4	0.585	0.331	0.352
10	0.459	0.331				5	0.585	0.333	
Mean.....		0.331			0.323			0.331	0.351
3	0.1627	0.116	1	0.1855	0.113	1	0.2059	0.116	0.117
4	0.1627	0.116	2	0.1855	0.114	3	0.2059	0.117	0.116
8	0.1627	0.116	8	0.1855	0.114	4	0.2059	0.116	0.117
10	0.1619	0.117				5	0.2059	0.117	
Mean.....		0.116			0.114			0.117	0.117

\* See Table I.

† d = inner diameter, from Table I.

methone, proceeded more rapidly in neutral than in acid solution, the more rapid reaction requiring 20 to 30 minutes when the mixture was warmed. Wadsworth and Pangborn (6) advocated a pH of 4.4 to 5 for the reaction and precipitation, and obtained complete precipitation in 4 hours at room temperature. Van Slyke, Hiller, and MacFadyen (12) advocated a pH of

5 to 6 and an overnight reaction at room temperature. Boyd and Logan (2) state that dimedon precipitation is not complete in less than 48 to 72 hours. The disagreement as to proper conditions for Vorländer's reaction called for an investigation of the effect of pH, and one carried out by using some method other than weighing the bis compound, which is susceptible to a marked influence of pH on solubility.

The method employed to determine the quantitateness of reaction of formaldehyde with Vorländer's reagent was the present chromotropic acid reaction. In tests associated with standardization of the chromotropic acid reaction, it was found that the bis compound was not hydrolyzed, and Vorländer's reagent yielded no formaldehyde, in the chromotropic acid reaction. To test whether dimethyldihydroresorcinol would react with formaldehyde under the conditions of the chromotropic acid reaction, a reaction solution was prepared by mixing, in order, 1 ml. of solution containing 75  $\gamma$  of HCHO, 24 ml. of the chromotropic-sulfuric acid reagent solution, 0.5 ml. of a solution containing 0.8 gm. of dimethyldihydroresorcinol in 100 ml. of buffer at pH 7.0, and enough chromotropic-sulfuric acid reagent solution to make a total volume of 50 ml. A control solution was prepared in the same way, except that water was substituted for the solution of dimethyldihydroresorcinol. Both solutions were heated in a boiling water bath for 30, 60, and 90 minutes, extinctions at 570  $m\mu$  being determined between the heating periods. The extinctions were identical. The amount of dimethyldihydroresorcinol tested, 4 mg., will be shown to be adequate for complete reaction with 150  $\gamma$  of HCHO. When the amount was increased to 10 mg., a reduction in extinction amounting to 2 per cent was caused. These results made possible a direct application of the chromotropic acid reaction to precipitation mixtures containing formaldehyde, Vorländer's reagent, and the bis compound.

The rates of reaction of formaldehyde with dimethyldihydroresorcinol at varied pH and at 37° are shown in Table V. The results were obtained by mixing 2 ml. of a solution containing 150  $\gamma$  of HCHO in McIlvaine's buffer at the pH indicated in Table V with 2 ml. of an aqueous solution containing 0.2 gm. of dimethyldihydroresorcinol per 100 ml., warming to 37°, and placing the mixture in an oven at 37° for a known time interval. Then a 2 ml. aliquot was tested by means of the chromotropic acid reaction. The results shown at zero time were obtained in the way described in the preceding paragraph, the extinctions observed after direct reaction of the HCHO solution with chromotropic acid being taken as 100 per cent. The pH values were determined electrometrically by means of a glass electrode; the only solution affected by HCHO was that designed to be pH 8.0 and found to be 7.7.

The times required for complete reaction at varied pH are given in bold-

faced type in Table V. If one assumes that the differences in directions as to time and pH previously published were due to differences in pH only, the results in Table V confirm the previous results, allowances being made for differences in temperature. The incompleteness of the reaction, 98.5 per cent, cannot be attributed to inadequate excess of Vorländer's reagent. In these tests the ratio of Vorländer's reagent to HCHO was 4000:150  $\gamma$ , or 27:1. In tests associated with standardization of the chromotropic acid reaction, the ratio was 2000:40 mg., or 50:1, and yet the reaction was found to be 98.0 per cent complete. Inspection of the Vorländer reaction solutions, which could contain not more than 1.5 mg of bis compound in 4 ml., or 370 mg per liter, revealed that the maximum precipitation oc-

TABLE V

*Rates of Reaction of Formaldehyde with Vorländer's Reagent at 37° and Varied pH*  
The values are given in per cent

Reaction time <i>hrs</i>	Formaldehyde determinable after Vorländer's reaction, in relation to that determined before Vorländer's reaction						
	pH 3.1	pH 3.5	pH 4.1	pH 5.1	pH 6.1	pH 7.0	pH 7.7
0*	100 0	100 0	100 0	100 0	100 5	100 0	101 0
	100 0	100 0	100 0	100 0	100 0	99 2	99 5
0 25	87 8		41 4	8 3	3 9	3 2	2 9
0 5	77 5		26 8	4 6	2 5	<b>0.9</b>	<b>1.5</b>
1	60 0	39 1	10 7	2 3	<b>1.9</b>	1 8	2 1
2			3 6	<b>1.4</b>	1 5	1 4	1 5
4	36 1	24 5	2 4	1 3	1 3	1 3	1 3
8	19 0	14 0	<b>1.5</b>	1 5	1 5	1 5	1 5
12	13 2	7 8	1 6	1 5	1 5	1 5	1 4
24	9 0	3.2	1 5	1 6	1 4	1 6	1 1
29	6 2	<b>1.5</b>					

\* See the text for the analytical conditions. The times required for complete reactions at varied pH are indicated by the values printed in bold-faced type.

curring at pH 5.1, and that a trace was precipitated at pH 7.7. For speed of reaction and for precision of sampling, pH 7 to 8 is preferable to more acid pH values in the present procedure for Vorländer's reaction, the reaction at pH 7 to 8 is at an end in 30 minutes at 37°.

*Transmethylenation from Compounds of Formaldehyde to Vorländer's Reagent*—The investigation of the reactions of formaldehyde with amino acids carried out by Wadsworth and Pangborn (6) revealed that arginine formed a compound which was most unstable in the presence of dimethyldihydroresorcinol. No matter how long the time taken for reaction of formaldehyde with arginine at pH 8 and 39°, samples taken for gravimetric estimation of free formaldehyde always and completely yielded to dimeth-



aldihydroresorcinol any HCHO combined with arginine, in 3 days at 39° and pH 4.4 to 5. The reaction of arginine with formaldehyde thus provided a means for testing transmethylenation under the present conditions of Vorländer's reaction.

A solution was prepared to contain 2.40 mM of arginine and 5.08 mM of formaldehyde in 300 ml. and buffered at pH 8. The solution was placed at 37° to be sampled from time to time. Prior to determining the amount of formaldehyde free in solution, each sample was diluted 1:10 with buffer.

TABLE VI

*Transmethylenation to Vorländer's Reagent from Arginine-Formaldehyde Compound*

Conditions of Vorländer's reaction (V.r.)			Formaldehyde measured spectroradiometrically		Formaldehyde reacting in V.r. (C = A - B)	f°	Formaldehyde bound to arginine, after V.r. (D = 50.8 - fC)	E†	Formaldehyde transmethylenated, in V.r. (F = $\frac{100(0.72-E)}{0.72}$ )
pH	Temperature	Time	No V r (A)	After V.r. (B)					

Arginine-HCHO reaction at pH 8, 5 days, 37°

	°C.		$\gamma$	$\gamma$	$\gamma$		$\gamma$	mole per mole	per cent
8.0	37	Few min.†	47.75	20.10	27.65	1.25, 1.15	17.6		
		15 "	47.75	15.10	32.65	1.029	17.21		
		30 "	47.75	14.75	33.00	1.015	17.31	0.72	0

Arginine-HCHO reaction at pH 8.0, 24 hrs., 37°

8.0	37	3 days	50.20	3.25	46.95	1.015	3.15	0.13	82
5.0	37	3 "	50.20	2.15	48.05	1.015	2.03	0.08	89
5.0	26	4 hrs.	49.65	10.05	39.60	1.015	10.61	0.44	39

\* Factor determined from HCHO left after Vorländer's reaction in the absence of arginine.

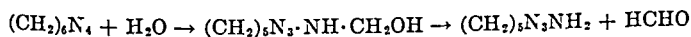
†  $E = (D \times 300 \times 10) / (1000 \times 30 \times 2.40)$ ; see the text.

‡ Warmed to 37° over a free flame, and immediately sampled for the chromotropic acid reaction.

Six different conditions for Vorländer's reaction were investigated. In three, the pH was kept constant at 8.0 and the temperature was 37°, but the time was varied from a few minutes to 30 minutes. The results are shown in the upper half of Table VI. With allowance for incompleteness of reaction at the different reaction times, there were two time intervals which were associated with the same estimation of HCHO bound to arginine, 15 and 30 minutes. Therefore it is assumed that no significant transmethylenation is likely to occur under the present conditions of Vorländer's reaction. When the pH, temperature, and time of reaction were varied,

transmethylenation did occur. These results are shown in the lower half of Table VI. Formaldehyde bound to arginine decreased from 0.72 mole per mole to 0.13 when the reaction time was prolonged from 30 minutes to 3 days. The decrease was to 0.08 mole per mole under conditions similar in every respect except that the pH was 5.0. Under the conditions advocated by Wadsworth and Pangborn (6), 4 hours at room temperature at pH 4.4 to 5, the amount of HCHO bound to arginine was 0.44 instead of 0.72 mole per mole. In the chromotropic acid reaction hydrolysis of the arginine-formaldehyde compound is very nearly complete, as shown by very little change in the amount of HCHO determinable by direct chromotropic acid reaction (no treatment with dimethyldihydroresorcinol).

Hexamethylenetetramine is also affected by transmethylenation through the action of dimethyldihydroresorcinol, but not significantly under the present conditions, unless hydrolysis has begun prior to association with Vorländer's reagent. Experiments were carried out in a manner similar to those on the arginine-formaldehyde compound, but the results are complicated by the fact that hexamethylenetetramine yields only 40 per cent of its available formaldehyde in the chromotropic acid reaction. The results are shown in Table VII, and reveal that about 2 per cent of formaldehyde in hexamethylenetetramine was transmethylenated to dimethyldihydroresorcinol when the solution containing hexamethylenetetramine had been hydrolyzed to an extent of 6 per cent in 2 days at pH 7.0 and 26°; prolonging the Vorländer reaction time increased hydrolysis to an extent equivalent to 22 per cent. Without hydrolysis, as in the freshly prepared solution, there was no transmethylenation. These results are consistent with the course of hydrolysis shown in the following equation,



which would be expected to be accelerated by removal of HCHO through the action of dimethyldihydroresorcinol.<sup>7</sup>

The incomplete yield of formaldehyde from hexamethylenetetramine in the chromotropic acid reaction, 40 per cent in 30 minutes, 70 per cent in 3 hours, is to be compared with a 92 per cent yield when hydrolysis is carried out in 9 to 10 M sulfuric acid, in the absence of chromotropic acid, for 1 hour and is continued for 30 minutes in a chromotropic acid reaction to determine the amount of formaldehyde evolved. The yield of formaldehyde is apparently not appreciably affected by the concentration of

<sup>7</sup> Transmethylenation may account for the 99 per cent yield of methylenebis(1,3-cyclohexanedione-5,5-dimethyl) from the reaction of hexamethylenetetramine with dimethyldihydroresorcinol. Ionescu and Georgescu (13) obtained this yield when the reaction was carried out for 10 minutes in hot water. So complete a yield in so short a time would not be expected in the absence of dimethyldihydroresorcinol, as the present results show.

sulfuric acid. Therefore, the low yield found in the chromotropic acid reaction must be attributed to the presence of chromotropic acid during the hydrolysis, and perhaps to a secondary reaction of chromotropic acid with hexamethylenetetramine or amines evolved from it by hydrolysis, a secondary reaction that may be similar to the general reaction of aryl-sulfonic acids with amines.

TABLE VII

*Transmethylenation to Vorländer's Reagent from Hexamethylenetetramine*

Hexamethyl- enetetramine solution	Time of Vorländer's reaction (V r.) at pH 7.0 and 37°	Formaldehyde reacting with chromotropic acid		Free HCHO + HCHO involved in trans- methy- lation (C = A - B)	Amount of free HCHO* ( $D = \frac{A - 102.3}{0.6}$ )	Formaldehyde involved in transmethylenation	
		No V.r. (A)	After V.r. (B)			Amount (E = C - D)	Proportion of amount possible ( $F = \frac{100E}{257}$ )†
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
Immediately after preparation .....	30 min.	102.3	101.8	0.5	0	0.5	0.2
After 2 days at pH 7.0, 26° .	30 "	112.3	89.3 91.1	22.0	16.5	5.5	2.2
	3 days	121.6	32.4 33.7	88.6	32.1	56.5	22.0
Theoretically possible..		257.0	0	257.0	257.8		

\* Assuming that hydrolysis begins by evolution of pentamethylenetetramine and HCHO, and that the 5-carbon and 6-carbon compounds are hydrolyzed at the same rate in the chromotropic acid reaction; viz., 40 per cent of HCHO in 30 minutes. If so,  $A = 0.4(257 - F) + F$ , where  $F$  is the free HCHO evolved by hydrolysis other than in the chromotropic acid reaction.

† 200  $\gamma$  of hexamethylenetetramine tested; this amount can yield  $(200 \times 6 \times 30)/140$ , or 257  $\gamma$  of formaldehyde, when completely hydrolyzed.

### *Applications to Biological Mixtures*

*Reaction of Formaldehyde with Amino Acids*—The combination of the chromotropic acid reaction and Vorländer's reaction lends itself to precise estimation of formaldehyde reacting with amino acids. The sensitivity of the chromotropic acid reaction makes possible quantitative estimation of microgram quantities of formaldehyde, quantities not easily determined by gravimetric estimation of methylenebis(1,3-cyclohexanedione-5,5-dimethyl). An example of this application is found in Table VIII, which presents results obtained from the reaction of formaldehyde with arginine at 37° and pH 8. The results are compared with those obtained by

Wadsworth and Pangborn (6) and are shown to agree well, except the 24 hour results. This difference may be attributed to transmethylenation, shown in Table VI to occur under the conditions employed by Wadsworth and Pangborn (6). The present results indicate that arginine reacts with formaldehyde to its fullest extent in 24 hours or possibly less time under the conditions tested.

*Treatment of Components of Chicken Eggs with Formaldehyde in Preparation of Vaccines*—An example of the application of the present method to problems of toxoid and vaccine preparation is shown in Table IX. These results<sup>8</sup> were obtained as follows: Allantoic fluid, chorioallantoic membrane, embryos, and yolks from a pool of chicken eggs after 13 days incubation were weighed, without preliminary drying, and mixed with formaldehyde

TABLE VIII  
Reaction of Formaldehyde with Arginine at pH 8 and 37°

Reaction time	Formaldehyde found		Formaldehyde reacting with arginine (2.40 mM)		Formaldehyde reacting with arginine	
	No Vorländer's reaction (A)	After Vorländer's reaction (B)	(C)*	(D)†	Present results (E = D/2.40)	Wadsworth and Pangborn (6) (F)‡
					mole per mole	mole per mole
days	$\gamma$	$\gamma$	mg.	mM		
0	50.80	1.85	3.33	0.11	0.05	0.07
1	47.75	15.55	54.99	1.83	0.76	0.63
4						0.74
5	47.75	14.75	51.93	1.73	0.72	
11						0.75

\* 2.40 mM of arginine mixed with 5.08 mM of HCHO in 300 ml. volume. This solution was diluted 1:10 before the final reactions with chromotropic acid. Therefore,  $C = ((50.80 - (A - B)1.015) \times 300 \times 10)/1000$  mg.

†  $D = C/30.03$ .

‡ Calculated from ((6) Table I) by  $\frac{148.0 - (\text{free HCHO})}{30.03} \div \frac{70.7}{14.0}$ .

in the proportion of 1 gm. to 7.51 mg. Prior to weighing, the embryos were ground in a Waring blender, and the yolks were shaken with glass beads. The mixtures were diluted with 5 volumes of McIlvaine's buffer solution at pH 7.0; one portion of each mixture was placed in an ice box at 10°; a second portion was placed in an oven at 37°. As controls for chromogenic material, similar mixtures were prepared without formaldehyde. At known time intervals, 1 ml. aliquots were diluted to 10 ml. with buffer at

<sup>8</sup> These components of chicken eggs were provided through the kindness of Colonel H. Plotz, Medical Corps, and Captain H. Hamilton, Sanitary Corps, of the Division of Virus and Rickettsial Diseases, Army Medical School.

pH 7.0. Then 1 ml. of the diluted mixtures was analyzed directly by the chromotropic acid reaction. 2 ml. of the diluted mixtures were mixed with 2 ml. of 0.2 per cent dimethyldihydroresorcinol solution, and Vorländer's reaction was carried out as described under the procedure. More precise results would have been obtained had not the biological considerations governed the choice of formaldehyde concentration, a 0.5 per cent "formalin" vaccine preparation; at least two-thirds of the added formaldehyde remained free even after 5 days at 37°, and this obscured the estimation of formaldehyde that had reacted with the biological material. It may be added that simple solutions of formaldehyde buffered at pH 7.0 and held at 37° were unchanged throughout these experiments.

*Reactions of Formaldehyde with Microorganisms*—For this purpose, a

TABLE IX

*Estimation of Amounts of Formaldehyde Reacting with 1 Gm. Quantities of Components of Chicken Eggs (Not Dried)*

Component of chicken egg (13 days incubation)	Temperature of reaction with for- maldehyde	Amount of formaldehyde reacting with component			Control for chromogenic materials
		24 hrs.	44 hrs.	120 hrs.	
	°C.	mg. per gm.*	mg. per gm.*	mg. per gm.*	mg. "HCHO" per gm.
Allantoic fluid.....	10	-0.02	-0.01	0.12	0.001
	37	-0.02	0.05	0.15	0.001
Chorioallantoic membrane ..	10	0.10	0.15	-0.01	0.012
	37	0.29	0.47	0.56	0.013
Embryo.....	10	0.47	0.36	0.64	0.003
	37	1.20	0.84	1.10	-0.001
Yolk.....	10	1.44	1.17	1.27	-0.001
	37	2.45	2.25	2.92	-0.004

\* 7.51 mg. of formaldehyde were added per gm. of component.

suspension of *Shigella sonnei* was chosen, and the amount of formaldehyde added was purposely kept down below the maximum required for reaction with the microorganisms. 1 ml. of a suspension in 0.9 per cent NaCl<sup>9</sup> (equivalent to about 6 mg. of nitrogen and to about 140 billion organisms) was mixed with 1 ml. of a solution containing 400  $\gamma$  of HCHO per ml. of 0.9 per cent NaCl. After 24 hours at 37°, 3 ml. of water were added to each of one pair of solutions, and 3 ml. of 0.2 per cent Vorländer's reagent in buffer at pH 7.0 were added to each of a second pair of similar solutions. 1 ml. of each of the diluted suspensions was analyzed according to the present procedures. (Despite the cloudiness of the suspensions, very

<sup>9</sup> Captain C. V. Seastone, Medical Corps, of the Division of Bacteriology, Army Medical School, was kind enough to provide the suspension of *Shigella sonnei*.

little error was caused in the spectroradiometric determinations by failure to filter through sintered glass; the difference in extinction so caused was 0.003.) Out of 400  $\gamma$  of HCHO mixed with the microorganisms, 17.9 were determinable by the direct chromotropic acid reaction, *A*, and 19.8 were determinable by the chromotropic acid reaction applied after Vorländer's reaction, *B*. The difference, *A* - *B*, was -1.9  $\gamma$ , or 0.5 per cent of the starting amount. Therefore, it is concluded that no formaldehyde was free in the suspension. The small difference, *A* - *B*, also indicates that trans-methylenation is not a factor in this instance. Furthermore, there was no change in *A* - *B* when Vorländer's reaction time was prolonged from 30 minutes to 24 hours. The 18 to 20  $\gamma$  of HCHO determinable after 24 hours reaction with the microorganisms can be attributed to hydrolysis of compounds formed by their reaction with HCHO. Prolonging the time of the chromotropic acid reaction from 30 to 90 minutes increased the yield of HCHO from 4.5 to 5 per cent to 11 to 12 per cent of a possible 400  $\gamma$ . Therefore, some of the formaldehyde was actually combined with the microorganisms. In view of the results obtained from hydrolysis of hexamethylenetetramine, a better estimate of the amount of HCHO bound to the organisms would be obtained by carrying out the hydrolysis in dilute acid in a Hamilton-Van Slyke reaction tube (3), and then determining the evolved formaldehyde by means of the chromotropic acid reaction.

*Assay of Vaccines, and Toxicological Applications*—The present procedures are readily adaptable to assays of vaccines. Four commercial preparations were analyzed in duplicate with the following results. Typhus vaccine A, 130.5, 131.6; typhus vaccine B, 134.0, 134.7; tetanus toxoid A, 384.8, 386.0; tetanus toxoid B, 578.4, 581.8  $\gamma$  of free HCHO per ml. of test solution. To obtain measurable extinctions at 570  $m\mu$ , the vaccines were diluted with an equal volume of water, and the toxoids were diluted with 9 volumes of water prior to the chromotropic acid reaction.

Determination of formaldehyde in tissues would seem to offer no great difficulties, because experiments on components of chicken eggs indicated that tissue chromogens did not interfere with the chromotropic acid reaction. Hemoglobin would interfere, but it and other proteins can be removed by precipitation (tungstic acid) and the filtrates can be analyzed directly by means of the present procedures. One source of error must be emphasized. When biological mixtures containing large amounts of sugars are submitted to the chromotropic acid reaction, the heating in 9 to 10 *M* sulfuric acid causes the reaction solution to take on a brown color. The color is probably due to a combination of humin formation, furfural production, and possibly charring. Interference from this source amounts to an extinction at 570  $m\mu$  equivalent to a formaldehyde concentration of 96  $\gamma$  per ml. of milk, and equivalent to a formaldehyde concentration of 7  $\gamma$

per ml. of blood plasma, when samples of milk and plasma were prepared to be free from protein. This source of error does not affect the quantitative recovery of added formaldehyde, but it does affect the concentration of formaldehyde which can be determined accurately by direct application of the chromotropic acid reaction. Thus, milk containing 1 mg. of HCHO per ml. can be analyzed with 10 per cent error by applying the chromotropic acid reaction to a protein-free filtrate after dilution of 1:100, the extinction at 570  $m\mu$  corresponding to 10  $\gamma$  of HCHO and 1  $\gamma$  of the non-specific "HCHO." The absorption curve produced by this non-specific "HCHO" is unaffected by the present Vorländer's procedure, so that combination of the chromotropic acid reaction with Vorländer's reaction permits of accurate estimates of HCHO in milk in amounts less than 1 mg. per ml.

Permanganate solutions neutralized by oxalic acid do not interfere with the chromotropic acid reaction. The oxidation of methanol to HCHO is being studied with a view to quantitative estimation of methanol by means of the present procedure.

*Determination of Glycine by Means of Formaldehyde Evolved in Ninhydrin Reaction*—The specificity of the chromotropic acid reaction for formaldehyde and the specificity of the ninhydrin reaction for amino acids shown by Van Slyke, Dillon, MacFadyen, and Hamilton (7) combine to make possible a specific method for estimation of glycine, since glycine is the only amino acid evolving HCHO in reaction with ninhydrin.

In the absence of other amino acids, glycine reacts with ninhydrin at pH 1 to evolve 0.81 mole of HCHO measurable in the present chromotropic acid procedure. Prolonging the chromotropic acid reaction time to 150 minutes increases the yield to a maximum of 0.96 mole of HCHO. This observation marks the first time that quantitative evolution of HCHO from glycine by the action of ninhydrin has been demonstrated. The details and results will be presented in a later paper.

#### SUMMARY

1. Eegriwe's chromotropic acid reaction (1) has been adapted to spectroradiometry. The extinctions vary with the concentration of formaldehyde according to Beer's law.

2. The reaction is carried out in 9 to 10  $M$  sulfuric acid by heating for 30 minutes in a boiling water bath, and is specific for formaldehyde. Amounts corresponding to 1.7 to 0.2  $\gamma$  per ml. of reaction solution can be estimated with 1 per cent error, by spectroradiometric measurement at a wave-length of 570  $m\mu$ .

3. Freshly resublimed hexamethylenetetramine was used to determine the extinction coefficient, and was found to be more convenient than conventional methods of standardization.

4. Conditions for condensation of formaldehyde with Vorländer's (5) reagent were found which permitted complete reaction with formaldehyde, without at the same time causing transfer of HCHO from labile compounds to Vorländer's reagent.

5. Free formaldehyde in biological mixtures was determined by the difference of HCHO determinable after Vorländer's reaction from that determinable before Vorländer's reaction. The biological mixtures included various components of chicken eggs, and suspensions of micro-organisms. Twenty analyses can be accomplished in 4 hours.

6. The specific ninhydrin reaction of Van Slyke, Dillon, MacFadyen, and Hamilton (7) can be combined with the chromotropic acid reaction for formaldehyde to provide a specific method for estimation of glycine. Glycine yields 0.96 mole of HCHO in reaction with ninhydrin at pH 1.

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# PEPTIDASE ACTIVITY OF LYMPH AND SERUM AFTER BURNS\*

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As early as 1914 Pfeiffer (1) observed an increase in the proteolytic activity of the serum of burn cases, which he interpreted as evidence that a toxin was produced at the site of the burn. In a more recent study on experimental burns, Leach, Peters, and Rossiter (2) called attention to the zone of damaged cells which surrounds the severely burned area as a possible site of origin of toxins. As these authors suggest, in the region of incomplete cell destruction it is likely that certain cell enzymes are denatured, while others are spared and may diffuse out of damaged cells, to the detriment or benefit of the animal as a whole. The present study was undertaken to investigate the possibility that proteolytic enzymes might be concerned in the post-burn process.

In searching for hypothetical substances which might diffuse out of damaged cells, it seemed likely that the lymph draining the burned area would provide a more concentrated source of the substances in question than the blood stream as a whole. Recent experimentation (3, 4) has shown that changes in the content of nitrogenous compounds and in the electrophoretic patterns of lymph and blood may occur in calves in response to burns.

The presence of proteolytic enzymes in lymph has been previously mentioned (5, 6), but little work on the nature of these enzymes has been done. More detailed study of these enzymes has been made possible by means of the techniques worked out by Bergmann, Fruton, and coworkers (7, 8).

## Materials and Methods

The substrates carbobenzoxyglycyl-L-phenylalanine and carbobenzoxy-L-glutamyl-L-phenylalanine were obtained through the courtesy of Dr. Max Bergmann. For the other substrates used, the authors are indebted to Dr. M. S. Dunn and the Amino Acid Manufactures, Los Angeles, California.

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The Grassmann-Heyde titration technique (9) was used in all the experiments. When the high concentration of protein in certain experiments interfered with the precision of the end-point determination in the 90 per cent alcoholic titration medium, the titration medium was subjected to centrifugation after addition of the alcohol, and an aliquot of clear supernatant solution was used for the titration. The pH of each experiment was checked by means of a glass electrode assembly.

Lymph protein levels were determined refractometrically, and good agreement was obtained with micro-Kjeldahl determinations run in addition on certain of the lymphs. The serum proteins were determined exclusively by the micro-Kjeldahl method of Wagner (10).

All dogs used in the experiments were anesthetized by means of sodium pentobarbital, 30 mg. per kilo, given intravenously. Rats were anesthetized by means of sodium pentobarbital, 40 mg. per kilo, given intraperitoneally.

#### EXPERIMENTAL

A search for proteolytic enzymes was made in peripheral lymph of dogs before and after burning the foot and ankle. Heparin was used in the cannula to prevent clotting, and flow was maintained prior to the burning by gentle massage of the foot. Over a period of 3 to 4 hours, a collection of several ml. of lymph was obtained, representing the pre-burn sample. Following the burn, the rate of lymph flow from the burned area increased, and massage was no longer necessary. 5 to 10 ml. of lymph were readily collected during the next 1 to 2 hours, and were used as the post-burn sample.

Collection of lymph was confined to a short post-burn time interval in order to avoid possible complicating effects due to multiplication of bacteria in the damaged area. Seitz filtration did not alter the enzymatic activity. The lymph was preserved on carbon dioxide snow unless enzymatic studies were performed immediately.

The presence of at least one enzyme capable of splitting peptide substrates was detected in normal lymph from the dog leg. The activity of this enzyme in the lymph from the leg consistently rose following burning of the foot and ankle by immersion in water at 90° for 10 to 15 seconds.

The possibility that increase in leucocytes in the post-burn lymph might account for this increase in enzymatic activity was first considered. There was no difference in the enzymatic activity, however, whether centrifuged or uncentrifuged lymph was used. The cell counts in lymph draining the burned area remained at low levels, even following the burn, as illustrated in Table I.

*Characteristics of Enzyme*—The following characteristics of the enzyme

responsible for splitting *l*-leucylglycylglycine have been found. It has a pH optimum at approximately 7.4, and does not require activation by reducing agents such as cysteine. Fig. 1 shows the relationship between pH and activity for the substrate *l*-leucylglycylglycine. As is evident from the curve, the activity of the enzyme in different buffers at a given pH is approximately the same. A pH dependence curve run without the addition of buffers, but with adjustment of the pH to the appropriate point by

TABLE I

*Cell Counts on Peripheral Lymph from Dog Legs before and after Burning Legs*

Dog No.	White blood cells		Red blood cells	
	Pre-burn	Post-burn	Pre-burn	Post-burn
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
B99	20	80	50	100
B100	100	260	280	240

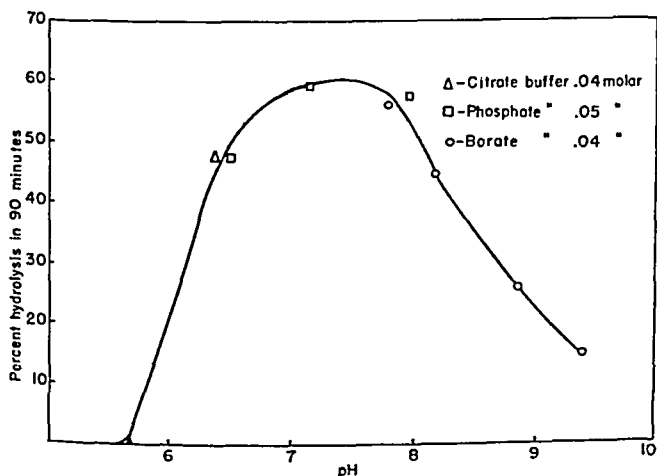


FIG. 1. pH dependence of hydrolysis of *l*-leucylglycylglycine by lymph

addition of acid or alkali, revealed a similar shape of curve and the same pH optimum. Since the activity of the enzyme in lymph, dialyzed for 6 days at 2.5° against changes of 1 per cent sodium chloride made up in glass-distilled water (although greatly reduced as a result of dialysis), is still present and is not increased by the addition of manganese, cobalt, or zinc in  $10^{-3}$  M concentrations, the enzyme differs from the leucylpeptidases described by Berger and Johnson (11).

The substrates *dl*-leucinamide, *l*-leucylglycine, and *dl*-alanylglycine are also split by lymph, the latter two at much slower rates than *l*-leucylglycylglycine. A rise in their activity in lymph likewise occurs following a burn. The question as to whether the same enzyme is responsible for hydrolyzing all four substrates has not been decided. Both normal and post-burn lymph are unable to split the following substrates, either at pH 5 or 8, with or without cysteine as an activator: carbobenzoxy-*l*-leucylglycylglycine, glycylglycine, benzoyl-*l*-argininamide, carbobenzoxy-*l*-glutamyl-*l*-phenylalanine, and carbobenzoxyglycyl-*l*-phenylalanine. In all cases the experiments were carried out at 40° for a 5 hour period, in the presence of toluene.

Since the hydrolytic process for *l*-leucylglycylglycine follows first order reaction kinetics and the hydrolysis invariably stops before 100 per cent calculated splitting of one peptide bond, it is reasonable to conclude that only one peptide bond is split. In certain experiments a rise in *K* occurred during the enzymatic reaction. Following dialysis of this lymph against 1 per cent sodium chloride the reaction usually followed first order reaction kinetics. Since *l*-leucylglycine is split by the enzyme, but glycylglycine is not, the hydrolysis probably occurs between the leucine and glycylglycine fractions of the molecule. Blocking of the terminal amino group of *l*-leucylglycylglycine by a carbobenzoxy radical results in complete loss of ability of the enzyme to hydrolyze this substrate carbobenzoxy-*l*-leucylglycylglycine. Blocking of the terminal carboxyl group in the substrate *dl*-leucinamide does not interfere with the activity of the enzyme; hence one may rule out the possibility that the enzyme is a carboxypeptidase. An imidoendopeptidase which has recently been found in extracts of intestinal mucosa by Smith and Bergmann (12) is also capable of hydrolyzing *l*-leucylglycylglycine, *l*-leucylglycine, and *l*-leucinamide. The activity of this imidoendopeptidase toward carbobenzoxy-*l*-leucylglycylglycine is not stated. The relative activity of lymph toward the above three substrates, however, is unlike that of the imidoendopeptidase toward these same substrates. In summary, the specificity requirements of the enzymatic activity in lymph best fit those of an aminoxoypeptidase, but one cannot be certain that more than one enzyme may not be responsible for splitting the substrates tested. An example of the reaction kinetics in the case of pre-burn and post-burn lymph on the same dog leg is given below in Table II.

*Change in Activity Following Burn*—A series of twelve experiments performed on dog lymph before and after burning the leg showed a significant rise in the activity of the peptidase following the burn.

Table III summarizes the findings of these experiments. It is to be noted that while the *K*, or activity of the enzyme per ml. of leg lymph, consistently increases following the burn, the *C*, or activity of the enzyme per mg. of protein nitrogen in the lymph, remains relatively constant. In other

TABLE II  
Reaction Kinetics of Lymph, Dog B56

Pre-burn*			Post-burn†		
Elapsed time	Per cent of substrate split	$K \times 10^3$	Elapsed time	Per cent of substrate split	$K \times 10^3$
min.			min.		
75	19	1.2	72	44	3.5
219	42	1.1	102	58	3.7
279	55	1.2	161	71	3.4
$C$			$C$		
$1.0 \times 10^{-3}$			$1.0 \times 10^{-3}$		

\* pH, beginning 7.98, ending 7.98;  $t = 40^\circ$ . Composition of test solution, lymph 1 ml., substrate and buffer 1 ml., toluene 0.01 ml. Concentrations in test solution, lymph 1.2 mg. of protein nitrogen per ml., *l*-leucylglycylglycine 0.05 M, borate buffer 0.02 M,  $K = 1/t \log a/a - x$ ,  $C = K$  per mg. of protein nitrogen per ml. of test solution.

† pH, beginning 8.05, ending 7.98;  $t = 40^\circ$ . Composition of test solution, lymph 1 ml., substrate and buffer 1 ml., toluene 0.01 ml. Concentrations in test solution, lymph 3.4 mg. of protein nitrogen per ml., *l*-leucylglycylglycine 0.05 M, borate buffer 0.02 M.

TABLE III  
Summary of Data on Enzymatic Activity of Dog Lymph toward *l*-Leucylglycylglycine before and after Burning\*

Lymph sample	$K \times 10^3$		$C \times 10^3$	
	Pre-burn	Post-burn	Pre-burn	Post-burn
B55	2.8	3.3	1.3	0.8
B56L†	1.3	3.1	1.0	0.9
B56R	1.4	3.5	1.0	1.0
B57R	2.0	4.8	1.5	1.6
B57L	3.7	5.8	2.3	1.9
B73	2.0	3.0	2.5	1.2
B74	3.3	2.3	2.5	0.9
B75‡	0.5	2.3	0.8	1.0
B79	1.0	2.7	1.1	1.0
B81	2.1	1.8	1.5	0.7
B97‡	0.4	1.4	0.6	0.7
B100	0.7	1.2	0.9	0.4

\* There was no hemolysis in either pre-burn or post-burn samples of lymph.

† The L and R refer to left leg and right leg in experiments in which both legs were burned.

‡ Lymph dialyzed 20 hours against 1 per cent sodium chloride at  $2.5^\circ$ .

words, the rise in enzymatic activity in the lymph from the leg area following the burn parallels the rise in protein concentration of the lymph.

Since the rate of flow of lymph from the leg following the burn increases 3 to 5 times, and since the enzymatic activity per ml. increases 2 to 4 times, the total enzymatic activity per unit time in the lymph from the burned area increases 6- to 20-fold.

If the formulation recommended for statistical analysis of small series is used (13), in which  $S = (X^2)/(N-1)$  and the standard error =  $(S)/(N)$ , the probability that the above changes in  $K$  are due to chance is  $<0.01$ . There is, then, greater than a 99 per cent probability that the change in  $K$  is a significant one. The change in  $C$ , however, is of doubtful significance statistically.

The enzymatic activity toward *l*-leucylglycylglycine in lymph decays if the lymph is allowed to stand overnight at 2.5°, or if it is dialyzed for a prolonged period against 1 per cent sodium chloride. Care was always taken, therefore, to treat the pre-burn and post-burn samples from a single animal in precisely the same way. It was possible to keep lymph frozen on carbon dioxide snow for a period of 1 or 2 weeks with little loss of activity. Storage over a period of months, however, resulted in a considerable decay.

Normal lymph obtained simultaneously from the two hind legs of the same dog has approximately the same level of activity in each leg no matter whether this activity is related to ml. of lymph or to mg. of protein nitrogen. The question arises as to whether the enzymatic activity toward *l*-leucylglycylglycine increases only in lymph from the burned area or whether there is a general increase in this enzymatic activity in lymph from other sources. In an effort to answer this question, the following experiment was performed.

Cannulas were introduced into the lymphatics in both hind legs of a dog and lymph was collected. One leg was burned for 15 seconds by immersion in water at 90°. The enzymatic activity in the lymph from the burned area rose markedly following the burn and remained elevated over a period of hours, during which time two separate collections of lymph were made, Sample 1 consisting of the lymph collected up to 2 hours post-burn, and Sample 2 the 2 to 4 hour post-burn collection. The enzymatic activity in the lymph from the unburned leg did not show the same degree of rise as it did in the burned leg, but a definite small increase in activity did occur. Table IV summarizes these results.

In a single experiment, lymph was collected from the leg of an anesthetized dog following the release of a muscle-crushing press applied for 5 hours, according to the technique of Duncan and Blalock (14). The rate of flow of lymph following release of the press did not increase appreciably. The enzymatic activity toward *l*-leucylglycylglycine (both the  $K$  and  $C$ ) did, however, increase 3- to 5-fold, indicating that direct trauma to skin and muscle can also evoke an increase in peptidase activity in the efferent lymph.

The peptidase activity in serum has been followed in two dogs, concomitantly with study of the level in lymph. Table V shows the result of this study, which indicates that the  $K_{LGG}$  of serum is higher than the corresponding level in lymph, but that the  $C_{LGG}$  of these two body fluids is similar.

**Human Burns**—A search for proteolytic activity was made in bleb fluid from burns of nine humans and bleb fluid from one human case of frostbite. The substrates *l*-leucylglycine, *l*-leucylglycylglycine, *dl*-alanylglycine, and *l*-leucinamide were split. The  $K$  for *l*-leucylglycylglycine ranged from  $1 \times 10^{-3}$  to  $11 \times 10^{-3}$ , indicating an 11-fold difference in enzymatic activity from one burn bleb to another.

TABLE IV

*Comparison of Enzymatic Activity of Lymph from Two Hind Legs of Dog B97 before and after Burning One of These Legs*

	$K_{LGG} \times 10^3$		
	Pre-burn	Post-burn, Sample 1	Post-burn, Sample 2
Burned.....	0.4	1.4	1.2
Unburned.....	0.2	0.3	0.3

pH 7.5;  $t$  40°; dialyzed lymph, borate buffer 0.04 M;  $K$  values averages of three closely agreeing readings in each case.

TABLE V

*Comparison of Enzymatic Activity in Lymph and Serum of Same Dogs*

Dog No.	$K_{LGG} \times 10^3$		$C_{LGG} \times 10^3$	
	Pre-burn	Post-burn	Pre-burn	Post-burn
B56. Lymph.....	1.4	3.5	1.0	1.0
" Serum.....	3.5	4.4	0.6	0.8
B100. Lymph.....	0.7	1.2	0.9	0.4
" Serum.....	2.8	2.8	0.6	0.6

**Calf Burns**—Through the cooperation of Dr. Cecil K. Drinker and Dr. Gertrude Perlmann, a comparison of the peptidase activity in calf sera was made before and after burning legs of anesthetized calves by complete immersion of both hind legs in hot water according to the method described by Glenn *et al.* (3). In six out of eight animals tested, the enzymatic activity of the serum had increased following the burn. Table VI summarizes these data. Statistical analysis of the data (13) reveals greater than a 95 per cent probability that they are not due to chance ( $0.05 > p > 0.01$ ).

**Rat Burns**—Three anesthetized rats were also burned by immersion of



70 to 80 per cent of the body in water at 95° for 30 seconds, and a comparison of the peptidase activities of serum before and after the burn was made immediately thereafter. In all three rats a small but definite increase in the post-burn enzymatic activity, both the  $K_{LGG}$  and  $C_{LGG}$ , occurred. The burns in the case of the calves and rats were more severe than those of the dogs, and were, therefore, theoretically more likely to bring out changes in the serum level of the enzyme following the burns.

*Peptidase Activity of Muscle, Skin, and Subcutaneous Tissue*—When dog muscle was ground and extracted with 2 volumes of 1 per cent sodium chloride, the supernatant solution following centrifugation had a  $C_{LGG}$  10

TABLE VI

*Enzymatic Activity of Calf Sera and Lymph toward l-Leucylglycylglycine before and after Burning*

	Calf No.	$K \times 10^4$		$C \times 10^5$		Serum protein	
		Pre-burn	Post-burn	Pre-burn	Post-burn	Pre-burn	Post-burn
						gm. per 100 ml.	gm. per 100 ml.
Serum	27	2.6	4.5	0.5	1.0	5.9	5.5
	35*	3.2	9.8	0.7	2.0	6.1	6.1
	25	6.1	9.3	1.4	2.1	5.5	5.5
	23*	8.6	7.4	1.8	1.3	5.8	6.8
	26*	5.1	6.7	1.0	1.4	6.4	6.0
	29	7.2	10.8	1.4	2.1	6.4	6.4
	24*	7.0	5.3	1.3	1.1	6.6	6.0
	31*	8.6	13.8	1.9	3.3	5.8	5.3
Lymph from burned leg	26-33 (Pooled)	2.9	3.7	1.2	1.2	Lymph protein	
						3.1	3.9

\* The post-burn sera of these animals were hemolyzed.

to 30 times that encountered in normal lymph. A similar extract of dog skin and subcutaneous tissue was made. A mixture of these tissues was taken from the shaved abdomen of a dog, cut into small pieces, homogenized with 2 volumes of 1 per cent sodium chloride in a Waring blender, and allowed to extract overnight at 2.5° after the addition of toluene. The supernatant fluid following centrifugation had a  $C = 13 \times 10^{-3}$ , about 10 times that found in normal lymph or serum.

*Activity of Erythrocytes*—The post-burn sera of certain calves (Table VI) showed hemolysis, amounting to 0.1 gm. per cent hemoglobin or less. Experiments were carried out, therefore, to determine whether hemolysis

of erythrocytes could liberate an enzyme capable of hydrolyzing *l*-leucylglycylglycine. Washed erythrocytes were hemolyzed by means of distilled water and centrifuged. The supernatant solution was found to have a  $C_{LGG}$  of  $3 \times 10^{-3}$ , and contained 4.4 gm. per cent hemoglobin. Part of the increase in the post-burn  $K_{LGG}$  of hemolyzed serum must therefore be ascribed to the hemolysis. It should be emphasized, however, that an increase in post-burn enzymatic activity was also observed in sera which showed no hemolysis. Table VII indicates the  $C_{LGG}$  of lymph, serum, bleb fluid, muscle, muscle exudate, skin and subcutaneous tissue, and erythrocytes. The muscle exudate was obtained as described by Aub *et al.* (15).

## DISCUSSION

Muscle, skin and subcutaneous tissue, and erythrocytes can theoretically

TABLE VII  
*Relative Enzymatic Activities toward l-Leucylglycylglycine*

Type of fluid	$C \times 10^3$
Lymph	0.6-2.5
Serum	0.6-0.8
Bleb fluid*	0.3-2.5
Erythrocyte extract	3
Muscle exudate	5
Skin and subcutaneous tissue extract	13
Muscle extract	21

\* Obtained from a human burn; all other fluids listed are canine in origin.

serve as sources of the peptidase activity found in lymph and serum. It is possible that the level of this enzymatic activity in lymph and serum may be an index of the rate of destruction of these tissues taking place in the body.

## SUMMARY

1 The presence of at least one peptidase, designated tentatively as an aminoexopeptidase, has been found consistently in normal lymph obtained from the legs of anesthetized dogs. An enzyme with similar properties has been found in serum and has been extracted from dog muscle, skin and subcutaneous tissue, and erythrocytes.

2 Following a burn or trauma to a dog's extremity, this enzymatic activity rises abruptly in the lymph draining the affected area. In the calf and rat an increase in this enzymatic activity in serum has consistently been found following a burn.

3. In bleb fluid collected from human burns, an enzyme with similar characteristics has been found.

The authors wish to express their indebtedness to Dr. John Graham for carrying out numerous surgical procedures, and to Dr. Joseph C. Aub for valuable suggestions and criticisms.

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# THE RESPONSE OF LACTOBACILLUS CASEI AND STREPTOCOCCUS FAECALIS TO VITAMIN B<sub>12</sub> AND THYMINE\*

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In 1939 Snell and Peterson published an abstract (1), and in 1940 a complete paper (2) concerning the occurrence, concentration, and properties of a growth factor required in the nutrition of *Lactobacillus casei* which they called the "norit eluate factor." The following year Hutchings, Bohonos, and Peterson (3) published further details regarding its purification and chemical properties, and reported that it was required by several lactic acid bacteria, including *Streptococcus lactis* R<sup>1</sup> (*Streptococcus faecalis*, American Type Culture Collection No. 8043). The same year Stokstad (5) reported the concentration from liver of an essential nutrient for *L. casei*, and Mitchell, Snell, and Williams (6) described a very potent concentrate from spinach, which they called "folic acid," essential for *Streptococcus faecalis* and also required for *L. casei*. Recently, Mitchell *et al.* (7-10) in a series of four papers have reported the concentration procedure for "folic acid" in detail, and have given information regarding its chemical and physiological properties. Pfiffner *et al.* (11), in 1943, reported the isolation in crystalline form from liver of an antianemia factor for chicks, and identified it as being the same as Snell and Peterson's "norit eluate factor." They called this compound vitamin B<sub>12</sub>.

Stokstad (12) later isolated the stimulatory compound from yeast and reported that, while the compounds from yeast and liver had equal potencies for *Lactobacillus casei*, the compound from yeast had only one-half the activity for *Streptococcus faecalis* as had the compound from liver. Keresztesy *et al.* (13) have reported another factor (SLR factor) from an unstated source that is potent for *S. faecalis* and not for *L. casei*. In a later paper Stokes *et al.* (14) state that the factor is changed by *S. faecalis* to a form active for *L. casei*. Recently Hutchings, Stokstad, Bohonos, and Slobodkin (15) reported the isolation from an unstated source of

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<sup>1</sup> In three recent papers Niven and associates (4) have shown that *Streptococcus lactis* R is an enterococcus, specifically *Streptococcus faecalis*. In conformity with this conclusion, the organism will be designated in this paper as *Streptococcus faecalis*. The organism is carried in the American Type Culture Collection under the No. 8043.

another crystalline compound which is active for *L. casei* and is also active for the nutrition of the chick. They use the term "*Lactobacillus casei* factor" to designate their compound. It was 85 to 90 per cent as active as that from liver when assayed with *L. casei*, but only 6 per cent as active as the liver compound by *S. faecalis* assay. More recently, Binkley *et al.* (16) reported that crystalline compounds isolated from liver and from yeast have equal potencies for *L. casei* and *S. faecalis*. Their compound from yeast was from a concentrate of a conjugate which had no activity for either organism, but had full activity for both organisms after enzymatic digestion.

Stokstad (5) found that for *Lactobacillus casei* he could partially replace his concentrate by guanine or other purines, and thymine. Stokes (17, 18) recently reported complete replacement of "folic acid" by thymine for *Streptococcus faecalis* and partial replacement for *L. casei*. In this paper we wish to report the growth response of *L. casei* and *Streptococcus faecalis* to vitamin B<sub>6</sub>, prepared both from liver and from yeast, and to thymine with or without the presence of vitamin B<sub>6</sub>.

#### EXPERIMENTAL

*Cultures and Inocula*—*Lactobacillus casei*, American Type Culture Collection No. 7469, and *Streptococcus faecalis*, American Type Culture Collection No. 8043, were used as test organisms. They were carried as stab cultures in a medium consisting of 0.5 per cent Difco yeast extract, 0.6 per cent sodium acetate, 1.0 per cent glucose, 2 per cent agar, and 0.05 ml. of mineral Salt Solutions A and B per tube (Table I).

The *Lactobacillus casei* inoculum was prepared by transferring from a stab culture to 10 ml. of liquid medium containing the same constituents, other than agar, as the stab medium. The culture was incubated at 37° for 24 hours. It was then centrifuged and the cells resuspended in 10 ml. of sterile water. 0.5 ml. of this suspension was transferred to another 10 ml. of sterile water. 1 drop of this second suspension was used to inoculate each tube in the assay series.

The *Streptococcus faecalis* inoculum was prepared by transferring from a stab culture to the basal medium used in the assay plus 10 millimicrograms of vitamin B<sub>6</sub>. The culture was incubated at 30° for 16 to 30 hours. It was then centrifuged and the cells resuspended in 10 ml. of sterile water. 1 drop of this suspension was used to inoculate each tube in the assay series.

*Media*—Two different media were used for *Lactobacillus casei*. The composition of the hydrolyzed casein medium is given in Table I. The hydrolyzed casein was prepared by hydrolysis of 50 gm. of Labco vitamin-free casein with 500 ml. of 12 N sulfuric acid for 24 hours in an autoclave

at 15 pounds pressure. The sulfate was removed by precipitation with 640 gm. of  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  in 400 ml. of boiling water. The precipitate was washed with 500 ml. of boiling water, filtered again, and the filtrates combined. The combined filtrates were adjusted to pH 3 with NaOH and the volume reduced to 1 liter by vacuum distillation. 5 gm. of norit A were then added, and the mixture stirred for  $\frac{1}{2}$  hour and filtered. The filtrate is designated as a 5 per cent solution of hydrolyzed casein.

The second medium was identical except for the nitrogen source. The amino acid mixture (2 mg. of each amino acid per tube) proposed by

TABLE I  
*Hydrolyzed Casein Medium for Lactobacillus casei*

Sodium acetate, %.....	0.6
Glucose, %.....	1.0
Casein, acid-hydrolyzed, %.....	0.5
L-Cystine, mg. %.....	10
L-Tryptophane, mg. %.....	2.5
Adenine sulfate, mg. %.....	2.0
Guanine hydrochloride, mg. %.....	2.0
Uracil, mg. %.....	2.0
Xanthine, mg. %.....	2.0
Riboflavin, mg. %.....	0.025
Calcium pantothenate, mg. %.....	0.05
Nicotinic acid, mg. %.....	0.25
Pyridoxine hydrochloride, mg. %.....	0.05
p-Aminobenzoic acid, mg. %.....	0.025
Thiamine hydrochloride, mg. %.....	0.05
Biotin, millimicrograms per tube.....	2
Salt Solutions A and B,* ml. per tube.....	0.05
Reaction of medium, pH 6.8.....	

\* Salt Solution A, 25 gm. of  $\text{K}_2\text{HPO}_4$  and 25 gm. of  $\text{KH}_2\text{PO}_4$  in 250 ml. of  $\text{H}_2\text{O}$ ; Salt Solution B, 10 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 gm. of NaCl, 0.5 gm. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.5 gm. of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  in 250 ml. of  $\text{H}_2\text{O}$ .

Hutchings and Peterson (19) as satisfying the amino acid requirements of *Lactobacillus casei* was used instead of the hydrolyzed casein, cystine, and tryptophane.

The medium used for the assay with *Streptococcus faecalis* was that described by Mitchell and Snell (20).

**Standards**—In all cases the standards were made up from the crystalline vitamin  $\text{B}_c$  preparation No. 41813 from Parke, Davis and Company.<sup>2</sup> The standard for *Lactobacillus casei* contained 0.2 millimicrogram per ml.; the standard for *Streptococcus faecalis*, 1.0 millimicrogram per ml.

<sup>2</sup> We are greatly indebted to Dr. A. D. Emmett of Parke, Davis and Company for the crystalline preparations of vitamin  $\text{B}_c$ .

*Procedure*—5 ml. per tube of double strength medium were used. The standard and other variables or samples were added to the tubes and the final volume in each tube made up to 10 ml. with distilled water. The tubes were plugged with cotton and autoclaved 15 minutes at 120°. After cooling, the tubes were inoculated with 1 drop of the appropriate inocula described above.

The *Lactobacillus casei* assay tubes of the hydrolyzed casein medium were incubated at 37° for 72 hours. Those containing the synthetic amino acid medium were incubated at 37° for 96 hours. After incubation the contents of each tube were titrated to the phenol red end-point with 0.1 N NaOH.

TABLE II

*Response of Lactobacillus casei to Crystalline Preparations of Vitamin B<sub>6</sub> from Liver and from Yeast\**

Vitamin B <sub>6</sub> per tube	0.1 N NaOH per 10 ml. medium					
	From yeast				From liver	
	Preparation 41813		Preparation 38613		Preparation 30003-A269A	
	8 runs	Average	6 runs	Average	3 runs	Average
millimicro- grams	ml.	ml.	ml.	ml.	ml.	ml.
0.0	1.4- 2.1	1.6	1.3- 1.8	1.5	1.4- 1.8	1.7
0.2	3.4- 3.9	3.6	3.1- 3.7	3.4	3.0- 3.3	3.2
0.3	4.3- 4.8	4.5	4.3- 4.8	4.4	4.1- 4.5	4.3
0.4	6.4- 6.8	6.5	6.0- 6.8	6.5	6.1- 6.4	6.3
0.5	7.0- 7.9	7.2	7.1- 8.1	7.3	7.0- 7.5	7.3
0.6	8.3- 8.8	8.5	8.6- 8.9	8.8	8.3- 8.7	8.6
0.8	9.0-10.1	9.4	9.0-10.1	9.3	9.1-10.1	9.3
1.0	9.3-10.1	9.8	9.2-10.1	9.9	9.1-10.1	9.7

\* Amino acid medium, incubated at 37° for 96 hours.

The *Streptococcus faecalis* assay tubes were incubated at 30° for 16 hours, at which time the turbidities were read in an Evelyn photoelectric colorimeter through a 660 mμ filter.

*Potency of Vitamin B<sub>6</sub> Preparations*—Two preparations of crystalline vitamin B<sub>6</sub> from yeast and one preparation from liver were furnished us by Parke, Davis and Company. Table II shows the range of titration values and the average value at the different levels of vitamin B<sub>6</sub> for *Lactobacillus casei* on the amino acid medium. No difference in potency between the preparation from liver and from yeast appeared, a result that is in agreement with the previous report of Binkley *et al.* (16). Table III shows the range of Evelyn readings and the average reading at the different levels of vitamin B<sub>6</sub> for *Streptococcus faecalis*. Again, there was

no difference in the potency of the preparations from yeast and from liver. This result does not agree with the report of Stokstad and associates (12), who found the yeast compound to be only one-half as active as the liver product. This disagreement may reside in some such difference as the type of yeast used, autolysis of the yeast, or action of yeast enzymes on precursors of the vitamin in processing the material. As already mentioned, Binkley *et al.* (16) isolated a substance that was inactive for both *Lactobacillus casei* and *Streptococcus faecalis* until it had been treated with enzymes. Unfortunately, neither Stokstad *et al.* (12) nor Binkley *et al.* (16) give any information as to how their compounds were prepared.

TABLE III

*Response of Streptococcus faecalis to Crystalline Preparations of Vitamin B<sub>12</sub> from Liver and from Yeast\**

Vitamin B <sub>12</sub> per tube	Evelyn colorimeter readings					
	From yeast				From liver	
	Preparation 41813		Preparation 38613		Preparation 30003-A269A	
	8 runs	Average	2 runs	Average	3 runs	Average
millimicro-grams						
0.0	90-92	91	90-92	91	90-92	91
1.0	84-87	85	86-88	87	84-88	86
1.5	80-83	81				
2.0	75-79	76	76-80	78	74-79	76
3.0	68-71	69	67-71	69	68-70	69
4.0	65-70	66	65-70	67	65-70	67
5.0	65-67	66	65-68	66	65-67	66

\* Medium of Mitchell and Snell, incubated at 30° for 16 hours.

The amount of vitamin B<sub>12</sub> needed to produce one-half maximum growth was not the same on the two media used for *Lactobacillus casei* (Fig. 1); with hydrolyzed casein medium it was 0.00042  $\gamma$ , a value which agrees fairly well with that reported by Luckey, Teply, and Elvehjem (21). On the synthetic amino acid medium a lower value, 0.00035  $\gamma$ , was obtained.

*Effect of Thymine*—The response of these two organisms to thymine was also investigated. Table IV gives a summary of these results. Thymine did not entirely replace vitamin B<sub>12</sub> in the nutrition of *Lactobacillus casei*; 3  $\gamma$  per tube gave a titration of 5.2 to 5.3 ml. of 0.1 N NaOH, but additional thymine did not always increase this titration and in some cases actually seemed to have an inhibitory effect on the acid production of the organism.

However, thymine completely replaced vitamin B<sub>12</sub> in the nutrition of *Streptococcus faecalis*, as has previously been reported by Stokes (17, 18).



20 $\gamma$  per tube of thymine produced an equivalent response to 5 millimicrograms per tube of vitamin B<sub>12</sub>.

The effect of added thymine on the standard curve was also noted, and is shown for *Lactobacillus casei* in Table V. It can be seen that the effect

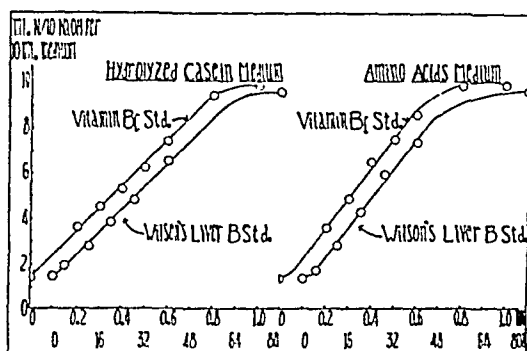


FIG. 1. Comparison of vitamin B<sub>12</sub> and Wilson's liver fraction B as standards for *Lactobacillus casei*.

TABLE IV

Response of *Lactobacillus casei* and *Streptococcus faecalis* to Thymine

Thymine per tube	<i>L. casei</i>		<i>S. faecalis</i>	
	0.1 N NaOH per 10 ml. medium		Evelyn colorimeter readings	
	Range of 5 runs	Average	Range of 4 runs	Average
$\gamma$	ml.	ml.		
0	1.2-1.8	1.5	90-92	91
1	2.0-3.4	2.7	88-91	89
3	5.2-5.3	5.2	84-86	85
5	5.4-6.4	5.9	78-81	79
8	5.5-5.9	5.7		
10	5.4-7.3	6.9	72-74	73
15	5.8-6.0	5.9		
20	4.8-5.9	5.3	65-71	68
100	5.8-6.9	6.3	63-65	64
200	3.6-7.3	4.9		

of the presence of only 1  $\gamma$  of thymine in a sample would be enough to give completely erroneous results on the amount of vitamin B<sub>12</sub> reported to be present in the sample.

The effect of thymine in a sample assayed with *Streptococcus faecalis* (Table VI) is not so marked, but erroneous results would nevertheless be obtained.

Assay of Wilson's Liver Fraction B and Difco Yeast Extract—Wilson's liver fraction B and dry powdered Difco yeast extract were assayed for their vitamin B<sub>6</sub> content with both organisms, and on two different media in the case of *Lactobacillus casei*. The materials were used as such without

TABLE V  
Response of *Lactobacillus casei* to Vitamin B<sub>6</sub> and Thymine\*

Vitamin B <sub>6</sub> per tube	0.1 N NaOH per 10 ml. medium				
	Thymine per tube				
	None	1 γ	5 γ	10 γ	100 γ
millimicrograms	ml.	ml.	ml.	ml.	ml.
0.0	1.8	3.4	5.4	5.6	6.8
0.2	3.9	6.9	7.6	8.3	9.2
0.3	4.8	7.7		9.0	
0.4	6.2	8.3	9.4	9.3	9.3
0.5	7.2	9.1		9.7	
0.6	8.8	9.2	9.7	9.8	9.6
0.8	9.1	9.6		9.8	
1.0	9.6	9.6			

\* Amino acid medium, incubated at 37° for 96 hours.

TABLE VI  
Response of *Streptococcus faecalis* to Vitamin B<sub>6</sub> and Thymine\*

Vitamin B <sub>6</sub> per tube	Evelyn colorimeter readings				
	Thymine per tube				
	None	1 γ	3 γ	10 γ	100 γ
millimicrograms					
0.0	90	88	84	72	63
0.5	87	83	81	70	64
1.0	84	83	78	65	63
1.5	82			64	62
2.0	75	78	74		
3.0	70		70		
4.0	65	62			
5.0	64				

\* Medium of Mitchell and Snell, incubated at 30° for 16 hours.

enzyme or other treatment. The results of these assays are reported in Table VII. These figures are the average of all values obtained. This includes averages at different levels in a given assay, and when more than one assay was run the average of the several runs. The departure of the highest or lowest values from the average is indicated by the plus or minus percentage figure. The two values obtained with *L. casei* in each case

agree within the limits of the experimental error in the assay procedure. In both cases the value obtained with *Streptococcus faecalis* was lower than that with *L. casei*. These figures may not give the actual potency of the materials, but it is probable that they are reasonably correct, since the values are so close together. It seems improbable that there can be much of the conjugated form of vitamin B<sub>c</sub> of Binkley *et al.* (16) in the yeast extract, since presumably the yeast has been autolyzed in the preparation of the extract. If there were much of the compound isolated by Keresztesy *et al.* (13) in the Wilson's liver fraction B, the figure by *Streptococcus faecalis* assay should be higher than that by *L. casei*.

Standard curves with both the vitamin B<sub>c</sub> preparation and Wilson's liver fraction B as standards for the two organisms are shown in Figs. 1 and 2. The curves in each case are almost parallel.

TABLE VII

Vitamin B<sub>c</sub> Content of Wilson's Liver Fraction B and Difco Yeast Extract (Water Extraction)

Organism	Medium	Wilson's liver fraction B		Difco yeast extract	
		Runs	Vitamin B <sub>c</sub> γ per gm.*	Runs	Vitamin B <sub>c</sub> γ per gm.*
<i>L. casei</i>	Hydrolyzed casein	4	11.9 ± 15	1	1.6 ± 10
" "	Amino acid	4	12.1 ± 10	3	1.7 ± 10
<i>S. faecalis</i>	Hydrolyzed casein†	3	9.6 ± 10	1	1.4 ± 15

\* The plus-minus figures are in per cent.

† The hydrolyzed casein is that described by Mitchell and Snell (20).

Luckey, Teply, and Elvehjem (21) compared the potency of highly purified preparations of folic acid, vitamin B<sub>c</sub>, and the *Lactobacillus casei* factor for *Lactobacillus casei* and *Streptococcus faecalis*. In terms of the quantities required for half maximum growth of the organism, vitamin B<sub>c</sub> was the most potent of the three.

*Hydrolyzed Casein As Nitrogen Source*—Hydrolyzed casein plus tryptophane and cystine is most often used as a nitrogen-source in assay media for the vitamins with *Lactobacillus casei*. Stimulatory effects of amino acids supplementing the hydrolyzed casein have been reported by Snell (22) and Dolby and Waters (23). Snell assumed the presence of 0.9 mg. of alanine in the amount of hydrolyzed casein added to each tube and found that the addition of 1 mg. more per tube was sufficient to produce all the stimulatory effect given by alanine. In our experiments the addition of 1 mg. of alanine per tube to the casein medium gave no increase in the titer of the blank, but did give approximately 10 per cent stimulation at the

various levels of vitamin B<sub>6</sub>. The only explanation we can offer for a lesser stimulatory effect than that obtained by Snell is a difference in the casein, or preparation of the casein hydrolysate, e.g. type of acid, degree of hydrolysis, or norit treatment. Addition of more alanine to our amino acid medium gave no stimulatory effect, but this was not surprising since this medium already contained 2 mg. of alanine per tube.

Dolby and Waters (23) reported a stimulatory effect upon addition of leucine, isoleucine, and threonine to their hydrolyzed casein medium when assaying with *Lactobacillus casei*, for a factor which may be related to vitamin B<sub>6</sub>. We found that the addition of these three amino acids

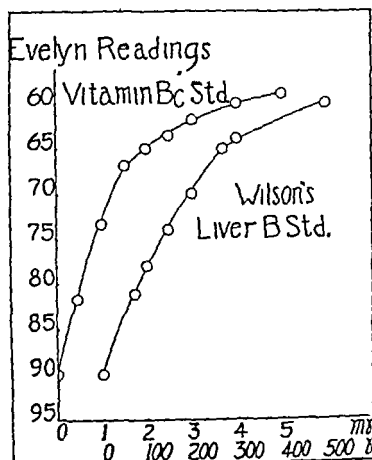


FIG. 2. Comparison of vitamin B<sub>6</sub> and Wilson's liver fraction B as standards for *Streptococcus faecalis*.

in the amount of 1 mg. per tube again resulted in an approximately 10 per cent stimulation in titer at the various levels of vitamin B<sub>6</sub>, but no stimulation at the blank. Again our hydrolyzed casein preparation was different from that used by Dolby and Waters.

It is evident, then, that hydrolyzed casein cannot be regarded as the best nitrogen source for optimum growth of *Lactobacillus casei*. As the hydrolysate is purified more and more to remove essential growth factors, the imperfections of the synthetic and semisynthetic media as compared with natural media become more apparent.

Fig. 3 shows the response of *Lactobacillus casei* to vitamin B<sub>6</sub> when various hydrolyzed caseins were used. Casein hydrolysates Samples A-1 and A-3 were received from another laboratory. Sample A-3, because of the high titration without added vitamin B<sub>6</sub>, was unsuitable for assay purposes.

The second curve, Sample A-3, "norited," gives the results after treatment of the hydrolysate with 1 gm. of norit-A per 10 gm. of casein at pH 3 for  $\frac{1}{2}$  hour with stirring. These curves show that an unsuitable casein hydrolysate may be made suitable by proper norit treatment.

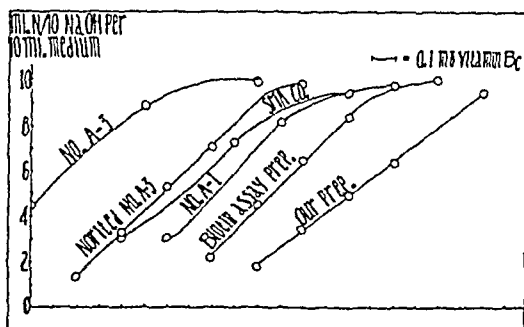


FIG. 3. Curves obtained with vitamin B<sub>6</sub> and various hydrolyzed casein preparations with *Lactobacillus casei*. For an explanation of the curves, see the text.

TABLE VIII

Comparison of Hydrolyzed Caseins Prepared at Different Times\*

Vitamin B <sub>6</sub> per tube	0.1 N NaOH per 10 ml. medium			
	Preparation I	Preparation II		Preparation III
	Run 1	Run 1	Run 2	Run 2
millimicrograms	ml.	ml.	ml.	ml.
0.0	2.1	0.9	1.2	1.4
0.2	3.7	3.2	3.6	3.2
0.3	4.6	4.3		
0.4	5.1	5.0	5.2	5.1
0.5	6.1	6.0		
0.6	6.4	6.8	6.8	6.9
0.8	7.6	7.9	7.9	7.6
1.0	9.1	9.0	9.2	9.1

\* Incubated at 37° for 72 hours (*Lactobacillus casei*).

The hydrolyzed casein used for the third curve was a commercial preparation for vitamin assays from the S. M. A. Corporation. The fifth curve was obtained with the casein that is described for use in the assay for biotin with *Lactobacillus casei* (24). The last curve is one obtained with the casein hydrolysate described above. It serves to illustrate that even curves with low blanks may still not be entirely satisfactory. It was this hydrolysate upon which the effect of the added amino acids was

observed. Moreover, the slope of this curve is not as steep as is usually obtained.

Table VIII illustrates the reproducibility of our hydrolyzed casein preparation. The difference in titration values between two preparations is no more than that between two runs.

#### DISCUSSION

Since so many forms of the factors for *Lactobacillus casei* and *Streptococcus faecalis* have been isolated or concentrated, assays vary with the standard and microorganism used. Mixtures of compounds of different potency for the two organisms may be present, and the resulting assays with the two organisms may not even show the same trend; *i.e.*, one organism may always show greater amounts, since compounds active only for one of the two organisms have been reported (13, 15). That this may be true has been indicated by Wieder (25) in his review. Binkley *et al.* (16) have also reported the occurrence and concentration of a conjugate, active in curing chick anemia but inactive for both organisms. The situation could be remedied if some treatment were possible which would convert all the compound to a form active for the given organism.

The effect of thymine is disturbing, especially in the concentration of some factor, since thymine may be concentrated as well. In the assays on Wilson's liver fraction B, however, thymine most likely does not interfere with the assay, since the greatest part of it would be bound in nucleic acid, which has been reported by Stokes (18) to have no potency for either organism.

#### SUMMARY

1. Vitamin B<sub>6</sub> isolated from yeast and from liver had the same potency for both *Lactobacillus casei* 7469 and *Streptococcus faecalis* 8043.

2. Thymine did not completely replace vitamin B<sub>6</sub> in the nutrition of *Lactobacillus casei*; it did replace it in the nutrition of *Streptococcus faecalis*.

3. As little as 1  $\gamma$  of thymine present free in a sample may erroneously influence the growth response of the test organism used in assay.

4. The vitamin B<sub>6</sub> content of Wilson's liver fraction B was found to be 12.0  $\gamma$  per gm. with *Lactobacillus casei* on two media, and 9.6  $\gamma$  per gm. with *Streptococcus faecalis*; of Difco yeast extract, 1.6  $\gamma$  per gm. with *Lactobacillus casei* on the two media, and 1.4  $\gamma$  per gm. with *Streptococcus faecalis*.

5. At 0.5 per cent level hydrolyzed casein did not supply quite enough amino acids to give optimum response of *Lactobacillus casei* in vitamin B<sub>6</sub> assays.

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## STUDIES ON CHOLINE ACETYLASE

### I. EFFECT OF AMINO ACIDS ON THE DIALYZED ENZYME. INHIBITION BY $\alpha$ -KETO ACIDS\*

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Recent investigations have provided evidence that the release and removal of acetylcholine (ACh) is an *intracellular* process connected with the nerve action potential. According to a new concept the active ester depolarizes the neuronal membrane by rendering it permeable to all ions. In a resting condition the membrane is selectively permeable to potassium. Flow of current is thus generated (action potential) which stimulates the adjacent region. There the process is repeated and the impulse in this way propagated along the axon (1-3).

The energy released by the breakdown of phosphocreatine is adequate to account for the total electric energy released by the nerve action potential. This could be shown with experiments on the electric organ of *Electrophorus electricus* (4). If the assumption is correct that the release and removal of ACh are the primary event in the alterations of the nerve membrane during the passage of the impulse, energy-rich phosphate bonds should be used for the restoration of the resting condition and the resynthesis of ACh. The phosphocreatine acts then as a storehouse for phosphate bond energy as in muscle (5), whereas adenosine triphosphate (ATP) acts as an intermediate link and is the primary source of energy.

Based on these findings, an enzyme, choline acetylase, could be extracted from brain which, in cell-free solution, in the presence of ATP under strictly anaerobic conditions synthesizes ACh (6). Some of the properties of the enzyme have already been described. The enzyme has active sulfhydryl groups which are readily oxidized and may be easily inactivated by moniodoacetic acid and Cu in low concentrations. On dialysis, the enzyme rapidly loses its activity (7).

The enzyme mechanism appears to be rather complex. Further studies on the properties of the enzyme, the intermediate reactions involved, the affinity of the enzyme to drugs, as well as its purification, will be described in this and in other papers to follow.

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### Methods

*Preparation of Enzyme Extracts from Fresh Brain*—The enzyme has been prepared essentially in the same way as described by Nachmansohn and Machado (6), with a few minor modifications. Brains of 6 to 8 week-old rats are rapidly weighed, cooled, and ground with silicate in 0.007 M phosphate buffer containing KCl in 0.05 M concentration. 5 ml. of buffer are taken per gm. of brain. The homogenizer described by Potter and Elvehjem (8) is used. During grinding, the suspension is cooled so that the temperature does not exceed 6–8°. The homogenized tissue is centrifuged at 2000 R.P.M. for a few minutes; the supernatant fluid is passed through gauze and then used for the experiments.

Choline chloride, sodium acetate, sodium fluoride, and physostigmine sulfate are added at about the same concentrations and in the same amounts as described. The total volume of all additions to the enzyme solution is about one-fourth of that of the solution. The final concentrations are for choline 0.0025 M, acetate 0.02 M, fluoride 0.02 M, physostigmine 0.0017 M. 3.5 ml. of this solution are put into a Warburg vessel. Further additions have usually been added in 0.5 ml. of fluid; otherwise 0.5 ml. of the original phosphate buffer is added to bring the amount in the main room of the vessel to 4.0 ml. so that all experiments are carried out in about the same volume. 0.3 ml. of ATP solution is put into the side bulb. When added to the main vessel, the initial concentration of ATP is  $3 \times 10^{-3}$  M, after experiments (described later) had shown that this concentration is close to the optimum.

The Warburg vessels are filled with  $N_2$  except those in which HCN is present. They are then put into a water bath at 37° and after 10 minutes, allowed for thermoequilibrium, the ATP is added to the main compartment. After an incubation period of usually 15 minutes, 0.8 ml. of 0.1 M phosphate buffer and then 0.3 ml. of N HCl are added to 3 ml. of the enzyme solution taken from the Warburg vessel. After 20 minutes, 0.3 ml. of N NaOH is added. The suspension is then centrifuged and the supernatant fluid is tested for ACh. If the solution is not tested the same day, 0.15 ml. of N HCl is added to 2.5 ml. of the supernatant fluid in order to bring the pH to about 4.5. At that pH ACh has optimal stability. A precipitate forms again which, after 10 minutes, is centrifuged. The supernatant fluid is left overnight in the refrigerator; the following day it is neutralized with 0.15 ml. of N NaOH and then tested.

*Preparation of Enzyme Extracted from Acetone-Dried Brain Powder*—Minced brain tissue is thrown into a Dewar flask containing acetone cooled with dry ice to –3° to –8°. After a few minutes, the acetone is poured with the tissue into a mortar and the tissue is thoroughly ground. The acetone is kept cold by the addition of pieces of dry ice. The tissue is separated from the acetone on a Büchner funnel and dried in air at room temperature.

A fine powder is obtained from which the enzyme may be easily extracted with the same phosphate buffer as is used for the extraction of fresh brain. 20 ml. of buffer are usually added per gm. of powder. The extraction is carried out in a mortar with the addition of silicate.

*Preparation of ATP*—ATP was at first prepared in the manner previously indicated. Later, the method of Kerr (9) was used, which was found to be more convenient and to yield larger amounts than the older methods. The ATP solution is prepared in the following way: 400 mg. of the barium salt are dissolved in 2 ml. of  $N$  HCl. 1.8 ml. of 0.5  $M$  sodium sulfate are added. The suspension is centrifuged and washed twice with 0.5 ml. of  $H_2O$ . The supernatant fluids are combined and neutralized with  $N$  NaOH. The concentration of this solution is about 0.045  $M$ .

The hydrolysis method of Lohmann (10) has been used for the determination of the ATP- $P_2O_5$ . The solution is hydrolyzed in  $N$  HCl. The difference between 7 and 30 minutes hydrolysis is subtracted from the difference between 7 and 0 minutes hydrolysis. The phosphate was determined by the method of Fiske and Subbarow (11) according to the modifications suggested by Lohmann (12). The photoelectric colorimeter of Klett and Summerson was used.

*Dialysis*—The enzyme is dialyzed in a cellophane tube against distilled water. The glass rod to which the cellophane tube is attached is bent so that the tube is fixed in a direction perpendicular to the rotating axis. The cellophane tube is immersed in the water bath with the rotating axis at an angle of  $45^\circ$ . In the cellophane tube is an air bubble which oscillates during rotation. In this way, not only the tube is rotated in the water, but the interior solution is continuously and thoroughly mixed by the bubble.<sup>1</sup> The dialysis is carried out in the refrigerator, although a few tests did not indicate a marked effect of temperature. In all experiments described in this paper, the enzyme has been dialyzed 120 minutes.

*ACh Determination*—The method of Chang and Gaddum (13) has been used as previously described, except that amounts in the range of 0.3 to 0.5  $\gamma$  of ACh only have been employed, at which the sensitivity of the frog's rectus muscle has been highest. The error does not exceed  $\pm 5$  per cent, but is usually smaller. All figures given in this paper are the average of at least two determinations. Only some of the low values (after dialysis) represent one determination; the error in this case is larger but obviously of less significance.

### Results

*Rate of ACh Formation*—In previous experiments (6, 7) the time of incubation of the enzyme with ATP was on the average 80 to 90 minutes. A

<sup>1</sup> We are indebted to Dr. Fritz Lipmann for suggesting this device.

study of the rate of ACh formation as a function of time reveals that the rate is highest during the first 15 minutes; it then decreases rapidly. A typical experiment is shown in Fig. 1.

The curve resembles that of many unstable enzymes, as for instance amino acid oxidase (14). Consequently, in the experiments reported in this paper, the time of incubation after addition of ATP was usually 15 minutes and the calculation of the amounts of ester formed per gm. of tissue per hour is based on the rate observed during the first 15 minutes. This amount varies, under the experimental conditions described, between 80 and 160  $\gamma$  but is generally 120 to 140  $\gamma$ . In larger and older rats with a brain weight of about 2 gm., the values obtained were lower than in young rats 6

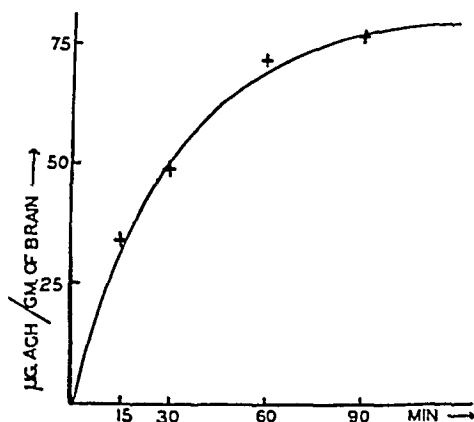


FIG. 1. Rate of acetylcholine formation as a function of time

to 8 weeks of age. In a few experiments with guinea pig brains the same values were found as with rat brains.

A few experiments carried out in air showed that the rate of ACh formation is markedly lower than that observed in strict anaerobic conditions. Addition of cysteine in 0.02 M concentration increases the rate of formation markedly; so that part of the inhibition by air may be attributed to the oxidation of —SH groups. For instance, in one experiment carried out in air, 74.5  $\gamma$  of ACh were formed per gm. per hour; with cysteine 115  $\gamma$  of ACh. In nitrogen without cysteine, 143  $\gamma$  were found.

*Optimal Concentration of ATP and Rate of Breakdown*—In spite of the presence of fluoride, ATP is decomposed at a rather high rate. This is shown in Fig. 2.

The optimal initial concentration of ATP under the experimental conditions employed is close to  $3 \times 10^{-3}$  M. Table I gives the figures of two ex-

periments with different concentrations of ATP. With  $2 \times 10^{-3}$  M ATP, the rate during the first 15 minutes is already as high as with  $4 \times 10^{-3}$  M. For a period of 30 minutes, there is more ACh formed at the latter concentration. No difference exists, however, at a concentration between 3 and 6

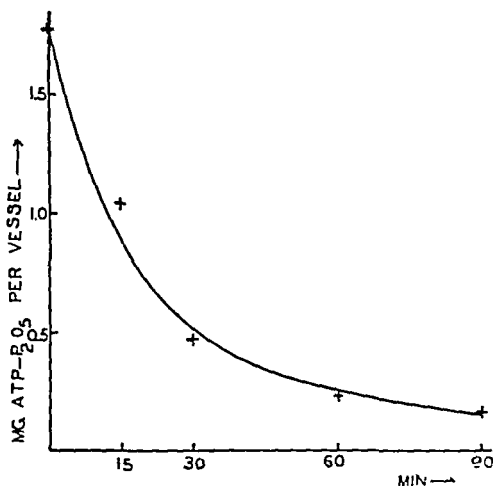


FIG. 2. Rate of adenosine triphosphate breakdown

TABLE I

*Effect of Concentration of Adenosine Triphosphate on Rate of Formation of Acetylcholine during 15 and 30 Minutes Incubation*

Concentration of adenosine triphosphate	Acetylcholine formed	
	15 min.	30 min.
$M \times 10^{-3}$	$\gamma$ per gm.	$\gamma$ per gm.
1	26.5	28.8
	24.9	29.2
2	30.4	43.5
	34.2	43.0
4	34.3	58.0
	31.0	49.0

$\times 10^{-3}$  M even for a period of 30 minutes, as was found in another experiment.  $3 \times 10^{-3}$  M ATP was therefore used throughout the experiments described in this paper.

Since between 3 and  $6 \times 10^{-3}$  M there is no difference in the rate of formation, the experiments indicate that the high rate of breakdown of ATP is not responsible for the rapid decrease of the enzyme activity. This was

also tested by repeated additions of ATP during the incubation period of 15 or 30 minutes, with Warburg vessels having two or three side bulbs. The rate of formation was not influenced by the repeated additions.

*Temperature Coefficient*—The effect of temperature on the activity of choline acetylase is demonstrated in Fig. 3, in which the rate of ACh formation is recorded at 37° and 29.5°. Calculated for a period of 15 minutes and a difference of 10°, the  $Q_{10}$  is about 2.0. It apparently falls with increasing duration, but this is probably due to the fact that at the lower temperature the enzyme is somewhat less rapidly inactivated than at 37°.

*Effect of Potassium*—The effect of potassium on choline acetylase was previously tested in a few experiments in the range between 0.02 and 0.06

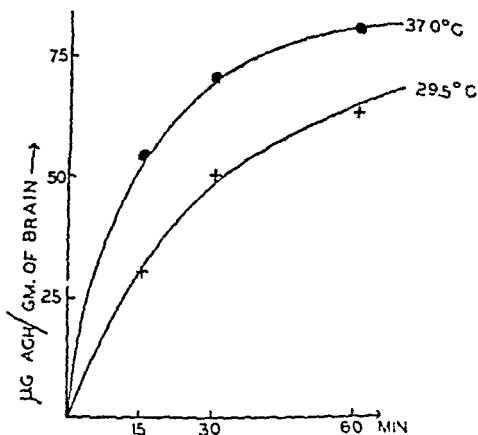


FIG. 3. Effect of temperature on acetylcholine formation

M. In this range, with the first enzyme preparations, which were less active than those obtained later, and with an incubation period of 80 to 90 minutes, no marked differences were observed.

If, however, the effect of potassium is measured over a wider range and for the period of optimal activity, the rate of formation rises markedly with increasing potassium concentration. The optimal concentration of potassium seems to be close to 0.08 M, as may be seen in Table II. This high optimum is interesting in view of the high potassium concentration found in nerves.

In the experiments described in this paper, the final concentration of potassium was 0.04 M, since at this concentration the potassium did not interfere with the determination of ACh on the frog's rectus muscle. On the other hand, 0.04 M appears to be sufficiently close to the optimum.

The importance of potassium is furthermore supported by the experiments with dialyzed extracts.

*Effect of Dialysis*—On dialysis, choline acetylase rapidly loses its activity. In 2 hours the activity decreases 80 to 85 per cent.

*Potassium*—Addition of potassium (final concentration 0.04 M) reactivates the enzyme only partly. It may increase from 15 to 20 per cent of the original activity to 30 to 45 per cent.

*Glutamic Acid*—Addition of glutamic acid also reactivates the enzyme. Only the natural *l*(+)-glutamic acid has an effect; *d*(-)-glutamic acid is practically inactive (7). The concentration required for the optimal effect is 0.02 M. 0.01 M is usually less effective, although in a few experiments the effect at this concentration was even slightly higher than with 0.02 M. In the presence of potassium, glutamic acid may reactivate the enzyme up to 80 per cent of the original activity. Fig. 4 shows an experiment in which

TABLE II

*Effect of K<sup>+</sup> on Rate of Acetylcholine Formation*

The figures indicate the amount of acetylcholine formed in micrograms per gm. per hour.

Concentration of K <sup>+</sup>	Experiment 1	Experiment 2	Experiment 3	Experiment 4
$M \times 10^{-2}$				
1	92	97	65	89
2	100	121.5	70	114
4	107	132.0	82	156
8	126	143.0	102	165
16	114	144.0	103	

such a strong reactivation has been obtained. It demonstrates also that the reactivation by glutamic acid is additional to that by potassium and that the effect decreases with falling concentrations. The combined effect of the two substances is, however, not always as strong as in this experiment. The reactivation varies between 50 and 80 per cent of the original, the average being 65 per cent. A series of experiments is reproduced in Table III.

The effect of glutamic acid, although decreasing with the concentration, may still be demonstrated at a concentration as low as 0.00125 M. This is of interest in view of the concentration possibly present in normal brain which may be of this order of magnitude (15). Results of two such experiments are reproduced in Table IV.

*Cysteine*—Of all the other amino acids tested only *l*(-)-cysteine has a stronger effect. This has obviously to be referred to the presence of the sulfhydryl group either protecting the —SH groups of the enzyme against oxidation or actively reducing oxidized groups. The activating effect of

cysteine is known for many enzymes containing —SH groups. The effect is present before dialysis, although much less pronounced. The optimal effect

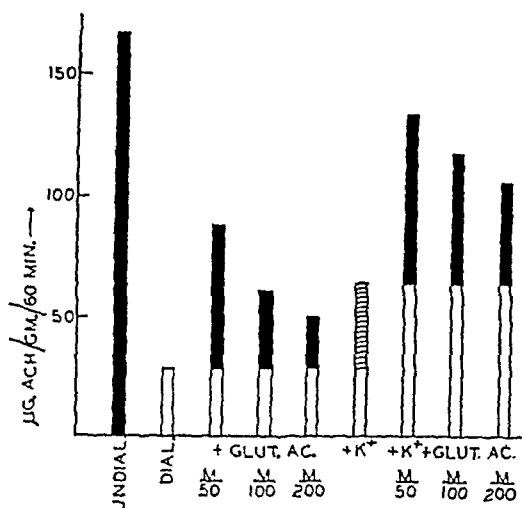


FIG. 4. Effect of *l*(+)-glutamic acid in varying concentrations on dialyzed choline acetylase without and with 0.04 M K<sup>+</sup>.

TABLE III

*Effect of K<sup>+</sup> (0.04 M) and of l(+)-Glutamic Acid (0.02 M) on Formation of Acetylcholine after Dialysis*

The figures indicate the amount of acetylcholine formed in micrograms per gm. per hour.

Experiment No	Undialyzed	Dialyzed		
			K <sup>+</sup>	K <sup>+</sup> + glutamic acid
1	122.5	17.5	38.0	72.5
2	154.5	23.5	61.0	100.0
3	136.0	19.0	53.0	97.5
4	165.0	28.0	63.0	132.0
6	129.5	22.0	48.0	78.5
7	124.0	23.5	45.5	73.0
8	136.0	20.5	37.0	70.0
9	154.0	24.0	54.4	94.0
Average.....	140.2	22.0	49.9	89.5

acid, the effect decreases rapidly with lower concentration. Two experiments are recorded in Table VI.

TABLE IV

*Effect of l(+)-Glutamic Acid in Varying Concentrations on Dialyzed Choline Acetylase*

The figures indicate the amount of acetylcholine formed in micrograms per gm. per hour.

	Concentration of glutamic acid	Experiment 1	Experiment 2
	$M$		
Undialyzed.....		124.0	141.5
Dialyzed.....		23.4	22.0
+ K <sup>+</sup> .....		45.5	55.0
+ " + glutamic acid.....	0.01	81.5	122.0
+ " + " ".....	0.005	73.4	89.0
+ " + " ".....	0.0025	69.2	66.6
+ " + " ".....	0.00125	61.2	63.5

TABLE V

*Effect of Cysteine (0.02 M) on Formation of Acetylcholine before and after Dialysis*

The figures indicate the amount of acetylcholine formed in micrograms per gm. per hour.

Experiment No.	Undialyzed		Dialyzed		
		Cysteine		K <sup>+</sup>	K <sup>+</sup> + cysteine
1	129.0		19.0	58.0	134.0
2	103.0	158.5	17.0	50.0	122.5
3	80.0	131.0	20.0	40.0	93.5
4	141.0	166.0	29.0	52.0	80.0
5	98.0	134.5	16.0	21.0	79.0
6	111.0	135.0	19.0	35.0	86.0
7	111.0	131.0	26.0	52.0	108.5
8	107.0	137.0	22.0	42.0	82.0
9	137.0	174.0	31.0	63.0	110.5
10	118.5	141.0	30.0	72.0	108.0
11	146.0	156.0	15.0	36.0	91.5
Average.....	117.0	147.0	22.0	47.0	100.0

Glutathione appears to be less effective than cysteine.

*Cyanide*—If the stronger effect of cysteine, compared with that of glutamic acid, is to be attributed to the reactivation of —SH groups along with the effect of the amino acid group, it would be expected that HCN might increase the effect of glutamic acid. Moreover the two compounds



combined might have an effect similar to that of cysteine. This is indeed the case. As an example, the values obtained in a typical experiment may be given. In the undialyzed extract 111.0  $\gamma$  of ACh were formed per gm. per hour, in the presence of cysteine 131.0  $\gamma$ . On dialysis, only 26.0  $\gamma$  were formed; in the presence of 0.04 M  $K^+$  52.0  $\gamma$ , of  $K^+$  + cysteine 108.5  $\gamma$ . On addition of glutamic acid 66.0  $\gamma$  were found, with HCN 77.0  $\gamma$ , with the two compounds together 94.0  $\gamma$ .

*Other Amino Acids*—The effect of glutamic acid and cysteine appears to be rather specific. All the other amino acids tested have either a small or no effect. Only *l*(+)-alanine has a marked effect, but still less than glutamic acid. In Table VII are summarized experiments with amino acids which have a small effect. No activation was found with the following:

TABLE VI

*Effect of Cysteine in Varying Concentrations on Dialyzed Choline Acetylase*

The figures indicate the amount of acetylcholine formed in micrograms per gm. per hour.

	Concentration of cysteine	Experiment 1	Experiment 2
	M		
Undialyzed.		118.5	
+ cysteine .	0.02	141.0	136.0
Dialyzed		29.8	28.6
+ $K^+$		72.0	62.0
+ " + cysteine	0.02	108.0	104.0
+ " + "	0.01	94.0	96.6
+ " + "	0.005	82.5	75.5
+ " + "	0.0025	78.0	65.8
+ " + "	0.00125		62.0

glycine, serine, aspartic acid, trypsin, histidine, methionine,  $\beta$ -alanine, and acetyl-*l*(+)-glutamic acid.

*Dicarboxylic Acids and Citric Acid*—No reactivation has been obtained with the following dicarboxylic acids: succinic, maleic, malic, and fumaric. Citric acid has a marked reactivating effect, almost as strong as glutamic acid. A few data are added to Table VII.

If potassium is not added to the dialyzed enzyme, several compounds which have no effect in the presence of potassium cause some reactivation. In the first observations on the dialyzed enzyme (7), potassium was not added. Since these effects are relatively weak and disappear in the presence of potassium, they seem of less interest.

*Inhibition of Choline Acetylase by  $\alpha$ -Keto Acids*—Acetylation of various compounds occurs easily subsequent to pyruvic acid oxidation. When

acetylation of choline was found in the presence of ATP, it was thought that pyruvic acid might be the precursor of acetic acid, acetyl phosphate being the intermediate.

TABLE VII

*Effect of Various Amino Acids and of Citric Acid on Dialyzed Choline Acetylase Compared to That of l(+)-Glutamic Acid (0.02 M)*

The figures indicate the amount of acetylcholine formed in micrograms per gm. per hour.  $K^+$  is always present in 0.04 M concentration.

Compound	Concentration	Experiment	Control	Control + glutamic acid
	M			
l(+)-Alanine.....	0.02	84.0	61.0	100.0
Citric acid.....	0.02	90.5	61.0	100.0
l(+)-Alanine.....	0.02	79.6	53.0	97.5
Citric acid.....	0.02	86.0	53.0	97.5
dl-Alanine.....	0.02	66.0	53.0	97.5
l(+)-Alanine.....	0.01	67.5	38.0	74.5
Phenylalanine.....	0.01	50.8	38.0	74.5
l-Tryptophane.....	0.008	56.2	38.0	74.5
".....	0.008	67.2	54.4	94.0
l-Proline.....	0.02	64.0	54.4	94.0
Valine.....	0.02	54.0	45.0	75.5

TABLE VIII

*Inhibition of Acetylcholine Formation by  $\alpha$ -Keto Acids in Extracts Prepared from Fresh Brain*

The amounts of acetylcholine found are given in micrograms per gm. per hour.

Concentration of $\alpha$ -keto acid	Pyruvic acid	$\alpha$ -Keto-glutaric acid	Concentration of $\alpha$ -keto acid	Hydroxyphenylpyruvic acid		Phenylpyruvic acid	
$M \times 10^{-3}$			$M \times 10^{-3}$				
Control	108.5	128.5	Control	155.0	103.0	131.0	134.0
0.312	63.5	101.0	0.125			118.0	
0.625	49.0	85.5	0.25	102.0	86.5	95.0	
1.25	51.5	54.5	0.5	93.0	72.0	86.0	93.5
2.5	47.0	44.4	1.0	98.0	58.5	52.0	59.0
			2.0	83.0	54.0		33.0
			4.0	68.0			

Surprisingly, however, strong inhibition was found in presence of pyruvic acid. When later it became evident that presence of an amino acid is required for the optimal activity of the enzyme system, it appeared of interest to determine whether other  $\alpha$ -keto acids, i.e. oxidized amino acids,

have an inhibitory effect similar to that observed with pyruvic acid.  $\alpha$ -Ketoglutaric acid and phenyl- and hydroxyphenylpyruvic acids have been tested so far. Table VIII shows that all these  $\alpha$ -keto acids have an inhibitory effect in concentrations of  $10^{-3}$  to  $10^{-4}$  M which is close to those which may occur in the cell. Rat brains were used; cysteine was not added.

No inhibition was found with acetoacetic acid.

*Separation of Choline Acetylase from Cholinesterase*—Extracts prepared from powder of acetone-dried guinea pig brain form about 400 to 900  $\gamma$  of ACh per gm. per hour. Since 1 gm. of powder may be obtained from 5 to 6 gm. of fresh brain, this rate of formation indicates the enzyme has lost only a fraction of its original activity by the treatment with acetone. This property is in contrast to that of cholinesterase, which was found by Nachmansohn and Lederer to be nearly completely inactivated by acetone (16).

TABLE IX

*Activity of Choline Acetylase, Prepared from Powder of Acetone-Dried Guinea Pig Brains, with and without Eserine, Fluoride, and Cysteine*

On the right side the effect of cysteine on such an enzyme preparation has been compared to that of glutamic acid and HCN. The amounts of acetylcholine formed are given in micrograms per gm. of powder per hour.

Compound	Experiment 1	Experiment 2	Experiment 3	Compound	
Control	470	583	820	Control	204
No eserine	440	303	780	Cysteine	645
" fluoride	440	583	810	Glutamic acid	405
" cysteine	180	226		HCN	512
				" + glutamic acid	533

Therefore choline acetylase may in this way be separated from cholinesterase. Addition of eserine has only a relatively small effect compared with that of extracts prepared from fresh tissue. Sometimes the effect is negligible, which indicates that the two enzymes are practically completely separated. In fresh extracts the rate of ACh formation is low compared with the rate of hydrolysis; the absolute amounts formed are small. No synthesis can be demonstrated without eserine in extracts prepared from fresh tissue in view of the high concentration of cholinesterase. Therefore it is not surprising that the presence of a small fraction of cholinesterase, after acetone precipitation, has some effect.

Adenosinetriphosphatase is also removed in these extracts prepared from powder. Addition of fluoride has no longer any effect. A few data are given in Table IX. Cysteine is necessary to obtain optimal activity with the extracts. Glutamic acid is less effective. HCN may reactivate the

enzyme rather strongly, but not as much as cysteine. One example is given in Table IX.

The enzyme solution prepared from dry powder is about twice as pure as that from fresh tissue. A solution prepared from powder which forms 1 mg. of ACh per hour contains about 0.3 gm. of protein. An enzyme solution prepared from fresh tissue and synthesizing the same amount of ACh per hour contains about 0.6 gm. of protein. Further purification of the powder is being carried out and will be described in a later paper.

The effect of  $\alpha$ -keto acids has been tested also on extracts prepared from dry powder of guinea pig brains. Cysteine was always added. A few data are given in Table X. It appears that in these more purified extracts the effect of  $\alpha$ -ketoglutaric acid is more pronounced than that of the other  $\alpha$ -

TABLE X  
*Effect of  $\alpha$ -Keto Acids on Choline Acetylase Prepared from Powder of Acetone-Dried Brain*

Compound	Concentration	Acetylcholine formed
	M	$\gamma$ per gm. per hr.
Control.....		600
Pyruvic acid.....	0.002	440
Control.....		390
$\alpha$ -Ketoglutaric acid.....	0.000125	200
"          ".....	0.00025	160
Control.....		645
Hydroxyphenylpyruvic acid.....	0.001	323

keto acids. This is of interest in view of the fact that  $\alpha$ -ketoglutaric acid is derived from glutamic acid.

#### DISCUSSION

The observations described in this paper have revealed several factors required for the optimal activity of choline acetylase. Potassium ions have to be present in a concentration close to 0.08 M, which is approximately that present inside the nerve cell. This is in contrast to cholinesterase, on which potassium has only a small effect, whereas the esterase requires divalent ions for its activity (17).

The effect of glutamic acid is of special interest, both from a theoretical and a clinical point of view. Cysteine, like glutathione, may enhance many enzyme systems. This effect is not surprising and has probably to be referred, partly at least, to the reestablishment of free —SH groups on the enzyme. The action of glutamic acid, however, appears to be specific, for it is difficult to assume that the effect is due to an action on the —SH groups.

If its sole function were to remove heavy metals, like Cu, other amino acids should be equally active. Moreover, the combination with cyanide supports the assumption of two different modes of action, since the effects of the two compounds are additive. The same is true for the fact that only the naturally occurring *l*(+) form is effective.

The precise rôle of glutamic acid in the enzyme mechanism has still to be explained. It may form a specific complex as an intermediate step, but there are other possibilities. It is surprising that aspartic acid, with a configuration so similar to that of glutamic acid, has no effect. This, again, is in favor of a very specific mode of action. The same applies to the difference between alanine and serine. The problem requires further investigation.

The interest attached to the action of glutamic acid is further emphasized by the inhibitory effect of the  $\alpha$ -keto acids. The fact that oxidized amino acids block the system may be due to a competitive reaction between  $-\text{CO}\cdot\text{COOH}$  and  $-\text{CH}\cdot\text{NH}_2\cdot\text{COOH}$  groups. This again points to a definite rôle of the latter group.

#### SUMMARY

The properties of the enzyme choline acetylase which forms acetylcholine (ACh) in strict anaerobic conditions in presence of adenosine triphosphate (ATP) have been further investigated.

1. In extracts obtained from rat or guinea pig brain, 100 to 150  $\gamma$  of ACh may be formed per gm. per hour. The optimal rate decreases rapidly after 15 minutes of incubation.

2. ATP is split, in spite of the presence of fluoride, at a rather high rate. An initial concentration of  $3 \times 10^{-4}$  M is optimal. At this concentration the nucleotide is not the limiting factor of the reaction.

3. The enzyme requires potassium ions. 0.08 M has been found to be near to the optimal concentration, which is approximately that found in mammalian brain.

4. On dialysis the enzyme becomes inactive. In 2 hours it has lost 80 to 85 per cent of its original activity. Addition of potassium reactivates it only partly. Further reactivation may be obtained with glutamic acid. Only the natural *l*(+) form is effective. The *d*(-) form has practically no effect. Cysteine is still more effective than glutamic acid and may reactivate the enzyme almost completely. Combined with cyanide, glutamic acid has an effect almost as strong as cysteine. Among all other amino acids tested, only *l*(+)-alanine enhances markedly the activity of the dialyzed enzyme, but not as much as glutamic acid. The other amino acids have either a weak effect or none.

5. Dicarboxylic acids have no effect on the dialyzed enzyme. Citric acid reactivates it almost as strongly as does glutamic acid

6.  $\alpha$ -Keto acids inhibit the enzyme in  $10^{-3}$  to  $10^{-4}$  M concentrations. Pyruvic, phenylpyruvic, hydroxyphenylpyruvic, and  $\alpha$ -ketoglutaric acids were tested. No inhibition was found with acetoacetic acid.

7. In extracts prepared from powder of acetone-dried brains, choline acetylase has lost only a small fraction of its original activity. Cholinesterase may be almost completely inactivated by treatment with acetone. In this way the two enzymes may be separated.

We are greatly indebted to the late Dr. M. Bergmann, to Dr. W. G. Bywater, Dr. V. du Vigneaud, Dr. D. E. Green, Dr. D. Shemin, and Dr. S. Ochoa for having given us some of the compounds tested.

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# THE IMPORTANCE OF "FOLIC ACID" IN RATIONS LOW IN NICOTINIC ACID\*

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After nicotinic acid was shown to be essential for the dog through the use of a modified Goldberger diet (1), Schaefer, McKibbin, and Elvehjem (2) succeeded in producing good growth in dogs fed a purified casein-sucrose ration supplemented with thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, choline chloride, and nicotinic acid. When nicotinic acid was omitted from this ration, drastic loss in weight, anorexia, and blacktongue developed in weanling puppies in 14 to 18 days, and in adult dogs in 30 to 45 days. Specific results on one of the experimental dogs showed that four separate doses of from 25 to 40 mg. of nicotinic acid resulted in an average gain in body weight equal to 34 gm. per mg. of nicotinic acid. Further work has shown that many dogs with blacktongue, particularly puppies, do not consistently respond to nicotinic acid therapy when this ration is used. Handler (3) has also observed inconsistent behavior of dogs on a purified ration and reports results in marked contrast to those seen earlier on Goldberger diets (4). Evidence is presented in this report that the "norit eluate factor" (Hutchings *et al.* (5)) or, as it is also known, "folic acid" (Mitchell *et al.* (6)) is a factor which needs to be considered in nicotinic acid-low rations.

## EXPERIMENTAL AND RESULTS

Weanling mongrel puppies, 6 to 8 weeks of age, were used in this study. The basal ration was essentially that of Schaefer *et al.* (2), except that the 3 per cent cod liver oil was replaced with cottonseed oil. The ration consisted of sucrose 66, cottonseed oil 11, acid-washed casein 19, and 4 parts of

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the generous supply of crystalline B vitamins; to the Abbott Laboratories, North Chicago, Illinois, for the generous supply of haliver oil; and to The Wilson Laboratories, Chicago, Illinois, for the solubilized liver extract, fraction L.

Dr. Mott Cannon kindly made the white cell counts and hemoglobin determinations reported.



Salt Mixture IV<sup>1</sup> (high manganese). The ration was fed *ad libitum* and each dog received in addition 100  $\gamma$  of riboflavin, 60  $\gamma$  of pyridoxine hydrochloride, 500  $\gamma$  of calcium pantothenate, and 50 mg. of choline chloride per kilo of body weight per day. The required amount of each of these vitamins was given in aqueous solution twice weekly. Halibut liver oil was given at the same time on the basis of 3 drops per kilo of body weight per week. This basal ration contained 0.64  $\gamma$  of nicotinic acid per gm. according to the microbiological method of Krehl *et al.* (7).

The following criteria were used to denote marked nicotinic acid deficiency: (1) drastic loss in weight, (2) anorexia, (3) inflammation of the gums with palatine redness, and (4) bloody diarrhea. When the animals became markedly deficient water consumption was greatly reduced.

TABLE I  
Vitamin B Content of "Folic Acid" Concentrate

Vitamin	Amounts found
	$\gamma$ per cc
Nicotinic acid	56.0
"Folic acid" (equivalent to pure vitamin B <sub>12</sub> )	7.8
Pantothenic acid . .	2.7
Pyridoxine	0.52
Biotin	0.036
Thiamine	0.20
Riboflavin .	1.7

One group of dogs was kept on the synthetic basal ration described above, and another group of dogs received in addition to this ration a "folic acid" concentrate, equivalent in "folic acid" activity to 1.0 gm. of solubilized liver extract. The procedure used in making the "folic acid" preparation was essentially that of Hutchings *et al.* (5), except that the preparation was carried only through the norit eluate stage. The original solubilized liver extract contained 11.5  $\gamma$  of vitamin B<sub>12</sub> per gm. by the *Streptococcus lactis* assay (8), and each cc. of the "folic acid" concentrate was equivalent to 7.8  $\gamma$  of pure B<sub>12</sub> by the same assay. It was necessary then to feed 1.5 cc. of this concentrate per dog per day, or actually 4.5 cc. were given every 4th day. The "norit eluate" was also assayed for nicotinic acid (7), pyridoxine (9), pantothenic acid (10), biotin (11), thiamine (12), and riboflavin (13), and the results are given in Table I. The amount of nicotinic acid contributed by this concentrate was not significant in view of the dog's requirement for this vitamin.

When a dog reached a state of severe nicotinic acid deficiency, 25 mg. of

<sup>1</sup> Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 109, 657 (1935).

nicotinic acid were administered in a capsule by mouth and the growth response noted. In this manner the dogs were standardized, each dog serving as its own control.

After the initial response to nicotinic acid, Dogs 1, 2, 3, and 5 (see Table II), those not receiving "folic acid," lost weight rather rapidly, and when 25 mg. of nicotinic acid were again given the dogs failed to respond, lost further weight, and died within periods of 2 to 7 days. Unsuccessful attempts were made to save Dog 2 by giving 65 cc. intraperitoneally and

TABLE II

*Response to Nicotinic Acid in Dogs Receiving Synthetic Ration and Same Ration Supplemented with "Folic Acid"*

Dog No. and sex	Ration used	Time required to produce deficiency	Amount nicotinic acid given	Weight response to nicotinic acid	Weight gained per mg. nico- tinic acid
		days	mg.	gm.	gm.
1 ♂	Basal	49	25	400	16
2 ♂	"	49	25	650	26
3 ♀	"	38	25	750	30
5 ♀	"	32	25	600	23
41 ♂	"	38	25	650	26
42 ♂	"	35	50	450	9
43 ♀	"	33	50	No response, death	
44 ♀	"	34	25		
8 ♂	" + "folic acid"	32	25	1450	58
9 ♂	" + " "	48	25	1850	74
12 ♂	" + " "	34	25	1000	40
16 ♀	" + " "	23	25	1100	44
34 ♂	" + " "	19	25	1000	40
40 ♀	" + " "	13	25	1100	44
45 ♂	" + " "	38	25	1200	48
47 ♀	" + " "	35	25	1350	54
38 ♂	" + " "	30	50	1500	30
39 ♂	" + " "	30	50	1550	31

35 cc. intravenously of a solution containing 5 gm. of dextrose, 2 mg. of thiamine hydrochloride, 2 mg. of riboflavin, 2 mg. of pyridoxine hydrochloride, 10 mg. of calcium pantothenate, and 25 mg. of nicotinic acid per 100 cc. respectively. Autopsies performed on these dogs invariably showed a marked intestinal enteritis, particularly severe along the upper tract, and a significant absence of intestinal contents. No other gross abnormalities were discerned.

Dogs that had received "folic acid" in addition to the synthetic basal ration developed nicotinic acid deficiency in an average time of 30 days as compared to 38 days for the other group. This difference may have little

significance because of the general overlapping in the two groups. In addition to the fact that dogs with blacktongue receiving "folic acid" responded considerably better to standard doses of nicotinic acid than those in the other group, they also were able to withstand repeated severe nicotinic acid deficiencies. The modified ration, therefore, is more useful in determining nicotinic acid in biological materials with the dog as the test animal.

Typical results obtained with two of the dogs, one on the basal ration and one on the basal ration plus "folic acid," are shown in Fig. 1.

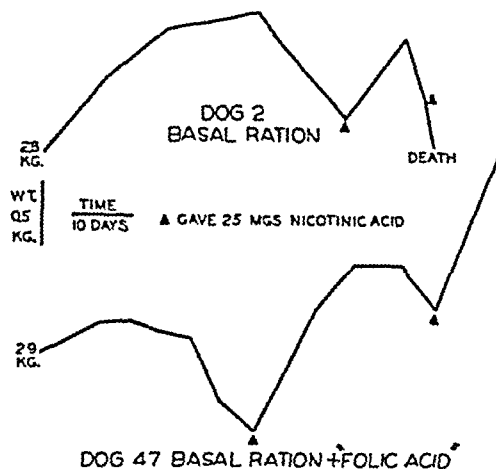


FIG. 1. Typical growth response obtained from nicotinic acid on the basal ration and the basal ration plus "folic acid."

It was observed that, in general, dogs receiving "folic acid" did not exhibit the severe mouth lesions so characteristic in classical blacktongue, although Dog 40 (see Table II) on a repeated deficiency demonstrated a most severe and extensive case of blacktongue. In addition, it was noted that many of the dogs receiving no "folic acid" developed a watery eye condition with considerable exudate. This condition was not observed in dogs receiving added "folic acid."

Since Handler and Dann (4) have reported white cell counts considerably below normal in cases of blacktongue produced on their purified ration and since it has been shown (14, 15) that "folic acid" is effective in counteracting sulfasuxidine-induced leucopenia in rats, it was deemed advisable to check the white cell count in the two groups of dogs under experiment. The white cell counts, made in duplicate from fresh blood drawn by ear puncture, were made at weekly intervals during various stages of the de-

ficiency. Hemoglobin values were determined in duplicate at the same time. These results are compiled in Table III.

Although there is considerable overlapping between the two groups, it is indicated that the dogs in the group receiving added "folic acid" have a tendency toward a higher white count. The low white cell count previously reported in nicotinic acid-deficient animals (4) was not observed in this experiment. Except for Dog 40 (see Table III), the hemoglobin

TABLE III  
*Blood Picture in Nicotinic Acid Deficiency*

Dog No. and sex	Ration used	Extent of deficiency*	Hemoglobin	White cell count
			gm. per cent	thousands per c.mm.
38 ♂	Basal + "folic acid"	+++	9.3	15.4
38 ♂	" + " "	++	10.9	12.4
39 ♂	" + " "	+++	11.1	19.9
39 ♂	" + " "	+	12.3	19.6
40 ♀	" + " "	0	14.1	14.1
40 ♀	" + " "	++	14.5	17.6
45 ♂	" + " "	+	9.8	11.4
47 ♀	" + " "	+	10.1	13.8
47 ♀	" + " "	+++	11.1	17.0
41 ♂	"	0	10.6	11.4
41 ♂	"	+++	8.7	9.8
42 ♂	"	+	9.8	10.2
43 ♀	"	+	9.6	9.8
44 ♀	"	+	10.0	13.9

\* Observed extent of deficiency when applying the criteria outlined in text. 0 = non-deficient, + = slight deficiency, ++ = moderate deficiency, +++ = severe deficiency.

levels were generally below normal, independent of the stage of the deficiency or the group examined.

#### DISCUSSION

The difference between the results herein reported and those of Schaefer *et al.* (2) may be explained at least in part by the fact that the deficiencies in this experiment were allowed to proceed to a greater degree of severity. Under the strain of severe deficiency additional complicating deficiencies may well play a part and the results reported indicate that "folic acid" is at least one factor involved.

It appears that "folic acid" or related compounds is synthesized in significant amounts by the intestinal bacteria and that dogs on a synthetic

diet containing the six B vitamins can depend upon this source of "folic acid." However, when nicotinic acid is restricted the degree of synthesis is greatly decreased but somewhat variable from dog to dog. The lack of food acting as a substrate in the tract during the nicotinic acid deficiency is undoubtedly an additional and complicating factor. Older dogs may or may not exhibit this deficiency, due to the fact that the intestinal flora may be too firmly established to be susceptible to alteration, or the storage of "folic acid" may be so great that no deficiency is observed.

The fact that the synthetic basal ration, supplemented with "folic acid," produces a greater and more consistent response to nicotinic acid under conditions of repeated deficiency improves the status of the biological assay for nicotinic acid, and assays of certain biological materials (16) by this method have checked very well with those by the microbiological method.

The observation that fewer dogs in the group receiving "folic acid" exhibit the severe mouth lesions might be explained by the postulation that folic acid prevents a complication of the nicotinic acid deficiency which is exhibited by this phase of the blacktongue syndrome.

#### SUMMARY

Young dogs placed on a nicotinic acid-low synthetic ration and allowed to proceed to a severe nicotinic acid deficiency responded poorly to standard doses of nicotinic acid and soon failed again, death generally ensuing despite administration of nicotinic acid. When the basal ration was supplemented with a "folic acid" concentrate derived from solubilized liver extract and the same experimental conditions followed, dogs responded adequately and consistently to nicotinic acid and could be used in repetitive assays.

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# THE EFFECT OF LIGHT ON THE STABILITY OF THE CARR-PRICE COLOR IN THE DETERMINATION OF VITAMIN A\*

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The Carr-Price reagent, a chloroform solution of antimony trichloride, has been widely used in the determination of vitamin A. The blue color developed upon mixing vitamin A and the reagent is unstable; fading begins immediately. The attempt to make photometric readings which are reproducible has led to a variety of practices in different laboratories. For example, galvanometer readings have been made at 3, 5, 15, 30, and 60 seconds after adding the reagent; the point of temporary stability has been used; curves have been plotted and extrapolated to zero time, while still others have employed photometers making use of condensers and ballistic galvanometers or photographic devices for measuring the color density. Some investigators have called attention to the difficulty of determining the point of temporary stability, although others have not found that this necessitates special consideration (1-7).

In this laboratory several different photometers have been available for determining vitamin A by the Carr-Price reaction. It has been observed that the stability of the blue color varied considerably when different instruments were used in the analysis. Since intensity of the light source appeared to cause the difference, this study was undertaken. Examination of the literature failed to reveal studies of the effect of light upon the Carr-Price reaction mixture.

These observations should be of interest to those using this method for the determination of vitamin A. The results should lead to a better understanding of the kinetics of the reaction, show why differences in the stability are often observed, contribute to a more exact determination of correction factors for the presence of carotenoids in the reaction mixture, and suggest a few factors to consider in the design of photometers for determining vitamin A by the Carr-Price method.

## *Procedure*

The Coleman universal spectrophotometer, model 11, with wave band set at 620 m $\mu$ , was used in this study. A resistance was placed in the circuit of the exciter lamp so that the brilliance of the light could be

\* Contribution No. 298, Department of Chemistry.



varied without dimming the light in the galvanometer unit. Matched 13 mm. square absorption cells were used. A vitamin A concentrate (Distillation Products, control, PC-2) was dissolved in U. S. P. chloroform and diluted to approximately 5  $\gamma$  per ml. After the usual preliminary adjustments of the galvanometer were made, an absorption cell containing 1 ml. of the vitamin A solution was placed in the light path and 9 ml. of 22.5 per cent antimony trichloride reagent (2) were added from a special pipette (8). The first reading of the galvanometer was made as soon as temporary stability was observed. Although varying slightly, depending upon the intensity of the exciter lamp, this point of stability occurred approximately 6 seconds after beginning the addition of the reagent. Further observations of the galvanometer were made at the time intervals shown in Fig. 1, with other intermediate intervals omitted from the figures to avoid crowding. The values shown are averages of several determinations. The intensity of the exciter lamp was adjusted to 13, 30, and 100 per cent of the full brilliance. The observations marked 0 per cent were taken by shifting the absorption cell containing the reaction mixture into the light beam (at 13 per cent of full brilliance) only long enough to read the galvanometer; generally this required about 3 seconds.

To study the effect of very strong illumination, the chloroform solution of vitamin A and the reagent were mixed and exposed intermittently to a 200 watt Mazda lamp at 3 inches distance. The absorption cell containing the reaction mixture was removed from the strong light only long enough to transfer it to the photometer and read the galvanometer.

The same stock solution of vitamin A was used to study the loss of color in the Evelyn and K W S Z photometers with the 620  $m\mu$  and 625  $m\mu$  filters respectively. The K W S Z was adapted for rapid reading by adjusting the setting on the decade box to the expected transmission. The reaction was carried out with the absorption cell in the photometer and any necessary adjustment of the transmission was made by reading the small galvanometer deflection which had been previously calibrated in terms of the decade box readings.

#### RESULTS AND DISCUSSION

The three photometers do not show identical galvanometer readings for the same vitamin A solution because of differences in construction and size of absorption cells. Therefore, to bring the results together and make comparisons as clear as possible, all readings have been transposed to apparent vitamin A concentration, by reference to the calibration curves previously prepared for each photometer based on the color obtained at the point of temporary stability. It should be noted that the curves in Fig. 1 also represent the relative color of the solution as it varies with time

following mixing of the reagent with vitamin A. The values shown for the Coleman photometer were all obtained by using the calibration curve prepared with the incident light at 13 per cent normal brilliance.

The data in Fig. 1 and Table I show clearly that light exerts a powerful effect in the fading of the blue color. As the incident light of the Coleman photometer is changed from 100 to 13 per cent of full normal brilliance, the rate of loss of color during the first minute decreases from 39 to 18.2 per

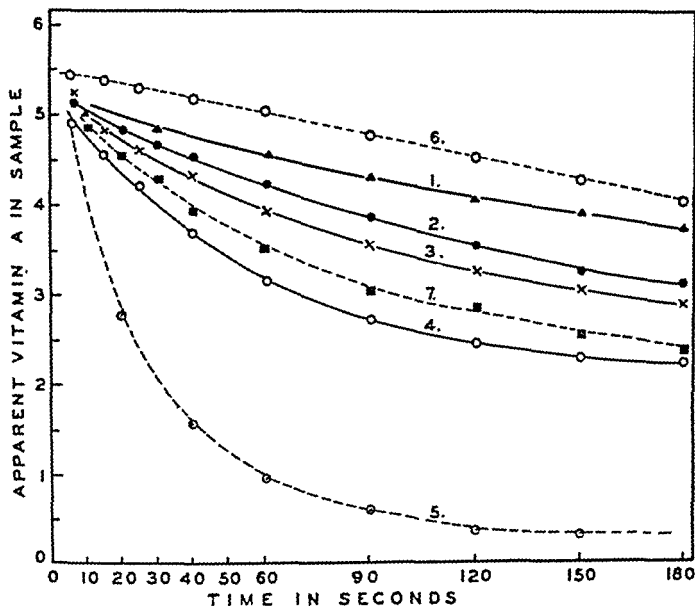


FIG. 1. Fading of Carr-Price color as a function of light intensity. Curves 1, 2, 3, and 4 are obtained with a Coleman photometer, when the reaction mixture is exposed continuously to 0, 13, 30, and 100 per cent of the normal photometer light intensity. Curve 5 shows the effect of exposure to direct illumination from a 200 watt incandescent lamp. Curves 6 and 7 represent normal fading obtained when the Evelyn and K W S Z photometers, respectively, are used.

cent. Exposure of the solution to light at 13 per cent of normal for only long enough to read the galvanometer reduces this loss of color to 11.7 per cent. The loss of color is 89.4 per cent if the light of a 200 watt bulb is allowed to strike the solution. A smaller loss, 7.8 per cent, is observed if light of low intensity such as that employed in the Evelyn photometer is used. The extrapolation of all curves to zero time tends to bring all the curves toward a common point. As would be expected from a study of the curves, there is a tendency for the photometric reading to be lower when

brilliant illuminations are used. The fading of a solution containing 10  $\gamma$  of vitamin A per ml. also was studied, with results essentially the same as shown in Fig. 1. The use of a perforated diaphragm to control the light intensity was tried, with results essentially the same as those obtained with rheostat control. Because of mechanical difficulties, the control of the light system by rheostats was adopted.

Although light will destroy vitamin A over a period of time, it was found that exposure of the vitamin A solution to the 200 watt light at 3 inches distance for 2 minutes before adding the antimony trichloride resulted in a blue color as high as that found for non-irradiated vitamin A. The light affects the blue color combination much more rapidly than it affects vitamin A itself. *The fading of the blue color appears to be due to radiations of the red region of the spectrum.* Ultraviolet light could have little if any effect in this case, since in one of the photometers used the incident light passed

TABLE I  
*Rate of Fading of Carr-Price Color from Initial to 60 Second Reading*

Photometer	Light intensity	Rate of color loss <i>per cent per min</i>
	200 watt Mazda lamp	89.4
Coleman	100% normal	39.0
"	30% normal	27.6
"	13% "	18.2
"	0 normal	11.7
K W S Z. . . .	Normal	32.6
Evelyn	"	7.8

through six pieces of glass and an aqueous solution before reaching the absorption cells; and yet fading was very rapid.

Hock (6) studied the kinetics of the Carr-Price reaction, using a photographic device to record color density. Fig. 4, a of his paper shows that vitamin A naphthoate fades so rapidly that if the same rate had continued for 70 seconds the value would have been practically 0 per cent of the maximum color developed. The results of this study indicate that a less intense light source would have caused less rapid fading. In making kinetic studies of the Carr-Price reaction, one might be led to assume either a zero or a first order reaction, depending upon the intensity of light employed in the photometer. Meunier and Raoul (9) studied the kinetics of the Carr-Price reaction of vitamins A<sub>1</sub> and A<sub>2</sub>. The intensity of the light source may cause the two forms of the vitamin to fade at different rates than those found in their study and merits further investigation. Since the determination of correction factors for the presence of carotenoids interfering

with the determination of vitamin A may be closely related to the kinetics of the reaction, this problem might profitably be reexamined, taking into account the effect of light upon the reaction mixture.

Dann and Evelyn (1) show that fresh and old solutions of antimony trichloride reagent cause fading to begin at different lengths of time after adding the reagent to vitamin A. An investigator using a photometer of different light intensity might erroneously conclude his reagent to be more or less stable than those prepared by Dann and Evelyn unless the effect of light is taken into account. The Evelyn photometer employs a minimal light source.

A study of these data indicates why some investigators find that observations made at the point of temporary stability are reliable, while others do not. With low light intensity the drift is so slow that one may obtain satisfactory reading over a period of several seconds, but with greater illumination the time at which the reading should be made is much more difficult to determine.

Measurements of vitamin A may be improved by using light of low intensity. It was found that satisfactory measurements of vitamin A could be made with the Coleman photometer when the intensity of the exciter lamp was decreased to less than 10 per cent of full brilliance. Under these conditions the calibration curve prepared approached a straight line, just as was the case when the instrument was used in the normal fashion. This method of adjusting the photometer has been adopted in this laboratory for the routine determination of vitamin A. However, calibration curves should be prepared for the light intensity to be used in subsequent determinations, since small differences in the readings are observed if the light intensity is varied from low to high illumination. This is probably due to slight differences in the character of the incident light and to changes in the rate of fading.

#### SUMMARY

1. The intensity of the incident light influences the rate of fading of the blue color developed in the Carr-Price reaction for vitamin A.
2. Investigations of the kinetics of the Carr-Price reaction should take into account the effects of the illumination in the photometer.
3. Photometers for determining vitamin A by the Carr-Price reaction should employ low intensity of incident light to reduce fading of the blue color to a minimum and make possible more precise determinations.

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# THE ACTION OF NITRITES ON BLOOD

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The interaction of sodium nitrite and blood takes place in three stages: (a) an induction period; (b) a reactionary period, usually rapid; and (c) a terminal period, often prolonged, during which the products of the reaction, chiefly methemoglobin, pass into hematin and degradation products. It is mainly with the first two periods that this paper deals.

Gamgee (1) concluded that the product formed from the action of nitrite on blood was a combination of the nitrite and oxidized hemoglobin. Sorby (2) and simultaneously Lankester (3) pointed out that the spectrum produced by the action of nitrites on blood was similar to that of methemoglobin described by Hoppe-Seyler in 1864. This view was eventually accepted by Gamgee (4).

One of us (C. R. M.), in a study of the pharmacological action of nitrites, complementary to a study of nitric esters in 1892, concluded that more than the formation of methemoglobin occurred in the interaction of sodium nitrite and blood, and later Haldane, Makgill, and Mavrogordato (5) showed that nitric oxide hemoglobin is one of the products of the reaction. Hartridge (6) revived the idea of a nitrite hemoglobin owing to the impossibility of obtaining the same spectrum from superposed solutions of nitric oxide hemoglobin and methemoglobin. Unfortunately he makes no mention of the amount or concentration of sodium nitrite used and this knowledge is material to the argument. Recently Barnard (7) has maintained that a compound of nitrite and methemoglobin is formed.

## Methods

The spectrophotometer employed in taking the curves illustrating this paper consisted of a large model Hilger constant deviation wave-length spectrometer having an average accuracy over the visible spectrum of about 1 Å, and a Nutting polarization photometer giving a triple field. The light source was a 100 c.p. pointolite lamp 63 cm. from the collimator slit. The width of this slit in most experiments was 0.05 mm. The ocular slit was 0.1 mm. wide and covered wave-lengths of 2.1 mμ in the

\* Much of the matter and all the figures illustrating this paper are taken from an M.D. thesis by one of us (W. M.) deposited in the University Library of Aberdeen in 1928.

yellow and 1.2  $\mu$  in the blue region of the spectrum. The internal diameter of the absorption cells was 1 cm. Unless otherwise stated, a screen of 5 per cent electrargol solution was placed in the beam of the polarized light to counteract the instrumental error inherent in spectrophotometric curves of most blood pigments and give them an appearance more in conformity with that visually observed. A dry electrargol screen of the type made by Ilford, Ltd., is now available.

The rate of reaction between nitrites and blood varies markedly between species ((1) p. 592), and such differences have indeed been noted between individual rabbits (8). Moreover, the addition of serum markedly affects the reaction velocity of nitrites on blood (9). In order to reduce blood variants as far as possible, experiments were made with crystallized oxyhemoglobin. That of the ox was found to be better adapted for the purpose than that of the horse or the hog and a method of preparing it in quantity was devised (10). The moist crystals are soluble in their own weight of water at 20° and are fairly stable. All the figures of this paper are from experiments with crystallized ox oxyhemoglobin.

The sodium nitrite used was purified by several recrystallizations from 90 per cent ethyl alcohol and was dried over concentrated sulfuric acid. Analysis showed it to be pure. Buffered solutions were used in most experiments, the mixtures employed being Walpole's acetate mixture to give pH 5.2, Sørensen's phosphate mixture to give pH 6.2 and 7.2, and Palitzch's borax-boric acid mixture to give pH 8.2 and 9.2. The pH of the solutions was corroborated by colorimetric methods with use of standard controls. At first attempts were made to determine the pH of the mixtures with quinhydrone and with hydrogen electrodes—the glass electrode was not available when these experiments were made—but the results were inconstant and it was found better to use buffered solutions and neglect the small and almost constant effect of the added oxyhemoglobin. For some experiments at neutrality tap water was used.

### *Reaction Velocities*

These experiments best show the course of the early phases of the action of nitrites on blood. The solutions of nitrite and oxyhemoglobin were intimately mixed, poured into an absorption vessel, and readings taken, usually every  $\frac{1}{2}$  minute, until the completion of the reaction. The earliest reading that could be observed was one 20 seconds after mixing the solutions. The readings were taken at  $\lambda$  577  $\mu$ . Change from oxyhemoglobin to methemoglobin is accompanied by a marked fall in absorption coefficient at this wave-length.

The results of the experiments are shown in Figs. 1 to 5. Each figure shows graphs of the reaction velocities of such concentrations of sodium

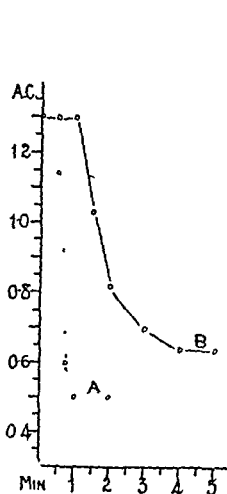


FIG. 1

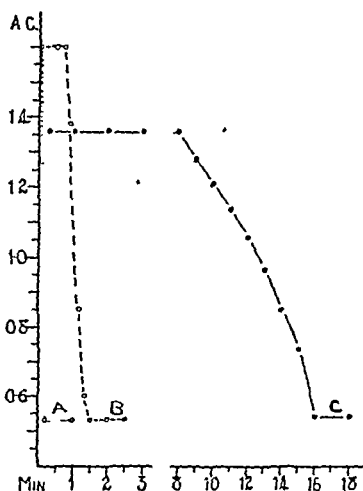


FIG. 2

FIG. 1. Reaction velocities of  $m/12,500$  and  $m/16,000$  (Curves A and B, respectively) sodium nitrite on oxyhemoglobin at pH 5.2. Ordinates, absorption coefficients; abscissae, time in minutes.

FIG. 2. Reaction velocities of  $m/1000$ ,  $m/3000$ , and  $m/7500$  (Curves A, B, and C, respectively) sodium nitrite on oxyhemoglobin at pH 6.2. Ordinates, absorption coefficients; abscissae, time in minutes.

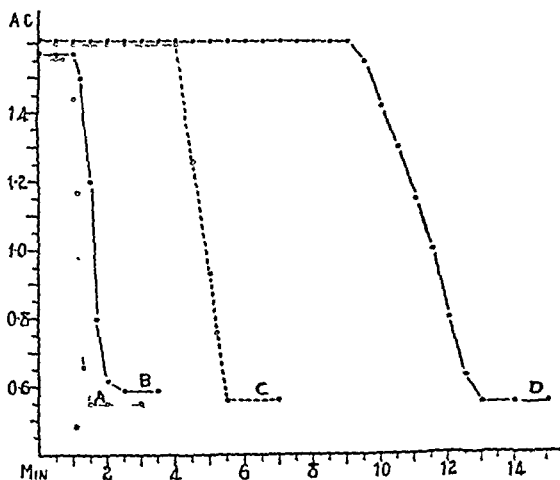


FIG. 3. Reaction velocities of  $m/500$ ,  $m/750$ ,  $m/1000$ , and  $m/2000$  (Curves A, B, C, and D, respectively) sodium nitrite on oxyhemoglobin at pH 7.2. Ordinates, absorption coefficients; abscissae, time in minutes.



nitrite as are within measurable limits on a moderately strong solution of oxyhemoglobin of definite pH. It will be seen that most of the curves have an induction period or lag followed by a reactionary period diminishing with increasing acidity or concentration of nitrite. Fig. 1 shows the reaction velocities at pH 5.2. The largest concentration of nitrite possible for observation was  $M/12,500$ . With this concentration the reaction began within 30 seconds and was complete in 1 minute. Larger concentrations of nitrite produced an almost immediate effect; the reaction

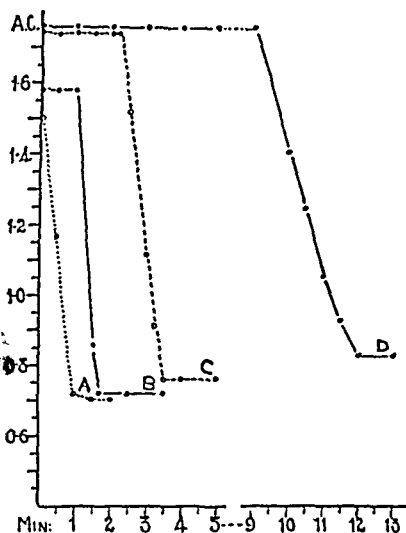


FIG. 4

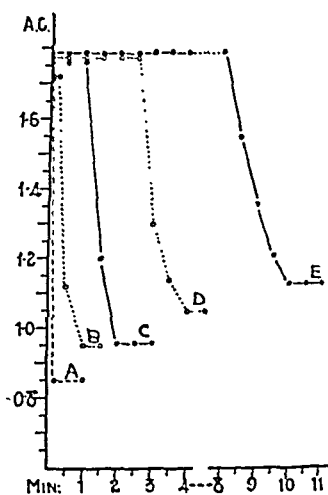


FIG. 5

FIG. 4. Reaction velocities of  $M/60$ ,  $M/120$ ,  $M/250$ , and  $M/500$  (Curves A, B, C, and D, respectively) sodium nitrite on oxyhemoglobin at pH 8.2. Ordinates, absorption coefficients; abscissae, time in minutes.

FIG. 5. Reaction velocities of  $M/6$ ,  $M/11$ ,  $M/25$ ,  $M/50$ , and  $M/100$  (Curves A, B, C, D, and E, respectively) sodium nitrite on oxyhemoglobin at pH 9.2. Ordinates, absorption coefficients; abscissae, time in minutes.

was over before any observation could be made.  $M/16,000$  nitrite did not produce any effect for 1 minute and the reaction period was slower. In Fig. 2 the graphs illustrate the effect at pH 6.2. At this pH  $M/1000$  nitrite has an almost immediate effect;  $M/3000$  nitrite shows a latent period of nearly 1 minute, followed by a rapid reactionary period; and  $M/7500$  nitrite has a long lag of 8 minutes and a reactionary period of approximately 8 minutes. At pH 7.2 (Fig. 3) similar effects are caused by  $M/500$ ,  $M/750$ ,  $M/1000$ , and  $M/2000$  nitrite. Figs. 4 and 5 give examples

of reaction velocities in pH 8.2 and pH 9.2 solution respectively, larger concentrations of nitrite being required with increase of pH.

The effect of pH on the reaction of nitrite and oxyhemoglobin is seen to be striking.  $M/1000$  nitrite has no demonstrable action on oxyhemoglobin in solution at pH 9.2 within a few hours of mixing, and has no effect on oxyhemoglobin in solution at pH 8.2 within half an hour; but it produces too sudden a reaction to be plotted on the scale adopted at pH 6.2 and 5.2. The experiments further show that according to the relative amounts of oxyhemoglobin and of nitrite used and the pH value, the reaction occurs at a definite time; and since something must be happening during the time interval it may be taken as an indication of a measure of activity. For the reaction to commence at a fixed time, it appears that as the pH diminishes the concentration of nitrite must be reduced according to a geometrical progression. Thus to begin 1 minute after mixing there is required at pH 9.2,  $M/25$  nitrite; at pH 8.2,  $M/120$  nitrite; at pH 7.2,  $M/625$  nitrite; at pH 6.2,  $M/3000$  nitrite; and at pH 5.2,  $M/16,000$  nitrite. The equivalent geometrical progression of  $a + ar + ar^2 + ar^3 \dots ar^n$ , where  $a = 25$  and  $r = 5$ , is 25, 125, 625, 3125, 15,625. A similar progression was found for a lag of 8 minutes. The association may be fortuitous but it emphasizes the great effect of pH; and the occurrence of a lag in the reaction suggests that nitrite does not act as such in transforming oxyhemoglobin to methemoglobin.

In this connection it is noteworthy that the velocity reactions of organic nitric esters (methyl nitrate, ethyleneglycol dinitrate, nitroglycerin) do not show an induction period. The curve of oxyhemoglobin transformation produced by them is similar in form to that of the hydrolysis of an ester.

### *Stationary Phase*

Darling and Roughton (11) say that, "The action of nitrite on hemoglobin is extremely complicated. It varies with the molecular ratio of nitrite to hemoglobin, pH, presence or absence of  $O_2$  and reducing agents, and possibly with other factors. Amongst the products of reaction found under varying conditions in vitro are methemoglobin, NO-hemoglobin and NO-methemoglobin." Our investigations suggest that the only compounds, apart from hematin and disintegration products not characteristic of nitrite action, are methemoglobin, acid or alkaline or a mixture of the two according to the pH, and, if the nitrite is in sufficient amount, nitric oxide hemoglobin. The nitric oxide methemoglobin described by Anson and Mirsky (12) as resulting from the action of nitric oxide on hemoglobin was not obtained by the action of nitrite; nor was the transient nitric oxide methemoglobin discovered by Keilin and Hartree (13) or the methemoglobin nitrite complex mentioned by Barnard (7).

The effect of nitrite was studied by varying one of the interacting factors of the possible combinations—the concentration of nitrite, of H ion, and of oxyhemoglobin—the other two being kept constant. The mixtures were prepared in the same way. A suitable solution of sodium nitrite was diluted to the required strength in the buffered solution employed and then the requisite amount of oxyhemoglobin solution was added. The mixture was well shaken and set aside in a corked vessel for at least 18 hours. The normalities given are those of the final mixtures.

Fig. 6 illustrates the effect of  $M/10,000$  sodium nitrite and  $M/12,000$  sodium nitrite on a concentration of 0.17 per cent oxyhemoglobin at neutrality. The stronger nitrite solution has produced practically complete conversion to methemoglobin, while the weaker nitrite still shows a considerable amount of oxyhemoglobin.  $M/10,000$  nitrite was too much for 0.13 per cent oxyhemoglobin. For a concentration of 0.26 per cent

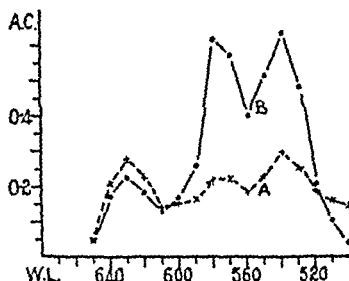


FIG. 6. Effect of (Curve A)  $M/10,000$  and (Curve B)  $M/12,000$  sodium nitrite on 0.17 per cent oxyhemoglobin in neutral solution. Ordinates, absorption coefficients; abscissae, wave-lengths in  $m\mu$ .

oxyhemoglobin  $M/6000$  sodium nitrite was necessary for complete conversion;  $M/8000$  sodium nitrite showed a fair quantity of oxyhemoglobin. For 0.33 per cent oxyhemoglobin  $M/6000$  sodium nitrite was insufficient and  $M/4000$  sodium nitrite sufficient for conversion to methemoglobin. The oxyhemoglobin solutions in these experiments were made by dissolving a known weight of moist crystals in the solvent, drying a weighed portion of the same batch to constant weight in a desiccator over sulfuric acid, and calculating the strength of the solutions from the data. If a molecular weight of 16,700 is assumed for hemoglobin, corresponding to a content of 1 atom of iron, the experiments suggest that the completed reaction of nitrite and oxyhemoglobin at these concentrations and pH is approximately molecular, a concentration of 0.17 per cent oxyhemoglobin requiring a concentration of  $M/10,000$  sodium nitrite. We do not wish to stress this molar relationship, although it has apparently been

accepted by several workers (Barcroft and Müller (14), Van Slyke and Vollmund (15), Vestling (16), von Issekutz (17)). Other investigators have found different ratios. Meier (18) regards it as molar only at pH 5; at neutrality he found 5 moles necessary for a complete change. Austin and Drabkin (19) give 0.5 to 0.7 mole of nitrite as effective in converting 1 mole of oxyhemoglobin; and Greenberg, Lester, and Haggard (20) also conclude that 0.5 to 0.6 mole of nitrite is sufficient but on an assumption that our experiments on reaction velocities do not seem to justify. If any molar relationship exists in this primary action of nitrites, it can only apply to what we have termed the stationary period and to specified conditions.

The studies on reaction velocities previously described show that the smaller the pH the less the nitrite required to change the oxyhemoglobin. This result was constant in the many absorption curves taken at various pH values. With minimal quantities of sodium nitrite for complete change of oxyhemoglobin to methemoglobin, the curves showed merely varying proportions of acid and alkali methemoglobin. At pH 6.2 only acid methemoglobin was formed; at pH 9.2 only alkali methemoglobin; at pH 7.2 and 8.2 both acid and alkali methemoglobin were formed, the acid methemoglobin predominating at pH 7.2 and the alkali methemoglobin at pH 8.2. In unbuffered solutions the concentration of the oxyhemoglobin, owing to the slight acidity of this substance, plays a part;  $M/10,000$  sodium nitrite added to solutions of 0.26, 0.56, and 1 per cent crystalline oxyhemoglobin in tap water was found to show an increasing amount of acid compared with alkali methemoglobin.

*Diffusion Experiments*—Some diffusion experiments which we made also show the great differences in the amount of sodium nitrite utilized at different pH values. 150 cc. of a strong solution of oxyhemoglobin in a parchment tube were dialyzed against 200 cc. of  $M/1000$  sodium nitrite solution, pH 6.3. The changes in the oxyhemoglobin were watched with the spectrophotometer. After 1 hour a trace of methemoglobin had been formed; after 22 hours most of the oxyhemoglobin had been converted to methemoglobin. The concentration of nitrite in the outer solution was, at 1 hour,  $M/1500$ ; at 22 hours,  $M/60,000$ . The outer solution was then replaced by fresh  $M/1000$  nitrite. In 3 days the concentration of nitrite had been reduced to  $M/5000$  and pure methemoglobin had formed. No further change in the pigment or in the concentration of nitrite due to dialysis occurred. Unfortunately the notes do not give the concentration of oxyhemoglobin in this experiment but it can be stated that the quantity of nitrite utilized was less than a quarter molecular. In two other experiments in which 10 cc. of a 20 per cent solution of oxyhemoglobin were placed in diffusion shells, which were put into 20 cc. of  $M/50$  sodium

nitrite buffered to pH 9.5 and 10.5 respectively, the following changes occurred: at pH 9.5, balanced state in 22 hours; concentration of nitrite  $M/80$ ; no further change: at pH 10.5, equilibrium not quite reached in 46 hours; concentration of nitrite at that time  $M/70$ ; balanced state reached in 70 hours; concentration of nitrite then  $M/80$ . In these two experiments 1.06 gm. of oxyhemoglobin had dissipated, at pH 9.5, 0.01194 gm. of sodium nitrite in 22 hours; at pH 10.5, 0.01132 gm. of sodium nitrite in 70 hours. Approximately 3 moles of nitrite had been utilized in each case. As the mixture at pH 9.5 showed unexpectedly some acid methemoglobin, the pH of the solution was taken; on the 6th day it was 8.3. That of the mixture at pH 10.5 had fallen to 9.8.

*Relative Excess of Sodium Nitrite*—With quantities of sodium nitrite much greater than are necessary to form methemoglobin at the pH of the mixture, an effect on the protein moiety of the hemoglobin, some destruction of the pigment and some formation of nitric oxide hemoglobin occur. Owing to these effects less methemoglobin is formed by large concentrations of sodium nitrite than by small. This result is shown in Fig. 7 which illustrates the effect of  $M/10,000$ ,  $M/1000$ , and  $M/100$  sodium nitrite in producing acid methemoglobin from oxyhemoglobin in solutions at pH 7.2 and 6.2.  $M/100$  sodium nitrite is seen to form less methemoglobin than  $M/1000$  solution at both pH values. In solution at pH 6.2 this concentration forms even less methemoglobin than  $M/10,000$  sodium nitrite.

The effect on protein and the destruction of pigment are not material to this paper but the formation of nitric oxide hemoglobin is of importance. In the absence of a reducing agent approximately  $M/100$  sodium nitrite is required to produce demonstrable quantities of nitric oxide hemoglobin from oxyhemoglobin. Nitric oxide hemoglobin is stable from pH 5.2 to 9.2. According to Drabkin and Austin (21) it is stable within the long range of pH 3.4 to 11.2. Since nitric oxide hemoglobin was only produced after the addition of much larger amounts of nitrite than were necessary to form methemoglobin, it seemed not improbable that the nitric oxide hemoglobin resulted from the action of excess of nitrite on the methemoglobin it had previously formed. This idea led to a study of the action of nitrite upon methemoglobin.

A solution of methemoglobin was made by adding a solution of potassium ferricyanide to a solution of oxyhemoglobin and dialyzing against running water to remove excess of ferricyanide. The effect of  $M/10$  and  $M/100$  sodium nitrite at pH 7.2 is seen in Fig. 8. Some transformation to nitric oxide hemoglobin has occurred. But even in  $M/10$  nitrite a considerable amount of methemoglobin remains unaffected. Concentrations much smaller than  $M/100$  nitrite have no effect at pH 7.2; so this concentration

may be regarded as the limit for the action of nitrite on methemoglobin at this pH. At pH 6.2 M/1000 seemed to be the limit of concentration. At pH 5.2 this concentration had only a slightly greater effect than at pH 6.2. At pH 9.2 M/100 did not produce nitric oxide hemoglobin. Thus the same large concentrations of nitrite are necessary to convert methemoglobin or oxyhemoglobin, even partially, to nitric oxide hemoglobin, and it would therefore seem probable that any conversion of oxyhemoglobin to nitric oxide hemoglobin occurs through the mediation of methemoglobin. Whether a prior reduction to hemoglobin is involved was not

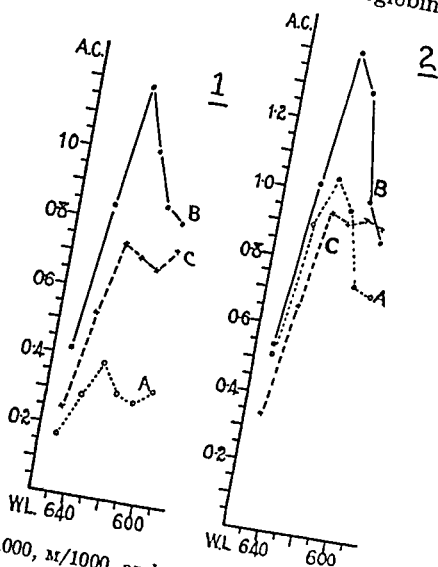


FIG. 7. Effect of M/10,000, M/1000, and M/100 (Curves A, B, and C, respectively) sodium nitrite on 0.26 per cent oxyhemoglobin solution at (1) pH 7.2 and (2) pH 6.2. No screen used. Ordinates, absorption coefficients; abscissae, wave-lengths in  $m\mu$ .

ascertained. We believe that it is, and apparently that is the opinion of Schmidt (22). Austin and Drabkin ((19) p. 80) appear to have obtained nitric oxide hemoglobin with smaller concentrations of sodium nitrite on solutions of dog blood corpuscles, since they state that the deviation "of the absorption curve with molar ratios of  $\text{NaNO}_2:\text{HbO}_2$  greater than 4:1 is such as would occur if the solution contained a mixture of  $\text{MHb}$  and  $\text{HbNO}$ ." Dog blood was not used in any of our experiments. Gamgee ((1) p. 592) found dog blood to be more susceptible to nitrites than ox or sheep blood, and such a difference may explain the discrepancy between Austin and

Drabkin's and our results. We doubt, however, whether the difference noted by Gamgee would apply to the crystallized oxyhemoglobin of the different animals; and it is to crystallized ox oxyhemoglobin that the curves of this paper refer.

*Reversible Reactions*—Equilibria are not uncommon in blood reactions. With nitrites they can be obtained for a time near neutrality, best on the alkaline side, with pigments having no dissociable oxygen. The effect of strong solutions of nitrite on methemoglobin has been referred to.

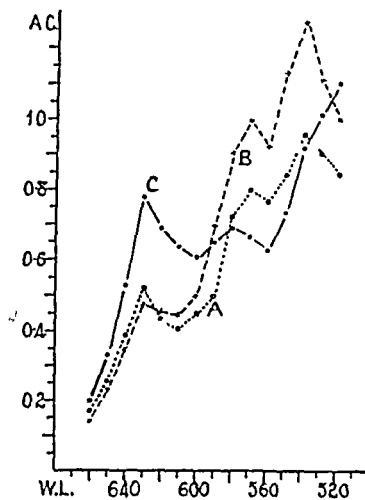


FIG. 8

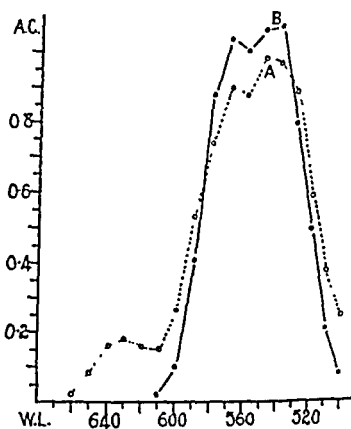


FIG. 9

FIG. 8. Effect of (Curve A)  $M/100$  sodium nitrite and (Curve B)  $M/10$  sodium nitrite on a solution of methemoglobin at pH 7.2. Curve C shows the absorption curve of the methemoglobin solution before the addition of the nitrite. Ordinates, absorption coefficients; abscissae, wave-lengths in  $m\mu$ .

FIG. 9. Curve A, effect of excess of sodium nitrite on a solution of NO hemoglobin after standing 24 hours. Curve B, NO hemoglobin solution used. Ordinates, absorption coefficients; abscissae, wave-lengths in  $m\mu$ .

Fig. 9 shows the effect of excess of nitrite on nitric oxide hemoglobin. The nitric oxide hemoglobin was prepared by exhausting a solution of oxyhemoglobin, washing with nitrogen, passing nitric oxide into the solution, and driving out the residual nitric oxide with nitrogen. Fig. 9 shows the formation of some methemoglobin 24 hours after the addition of the nitrite, demonstrating that the action is reversible. It is comparable with the action of a similar concentration of nitrite on oxyhemoglobin.

*Effect of Reducing Agents*

By the combined use of sodium nitrite and almost any substance which will reduce oxyhemoglobin to hemoglobin, nitric oxide hemoglobin may be produced. Haldane, Makgill, and Mavrogordato (5) and Hartridge (6) employed ammonium sulfide. We have generally used sodium hydro-sulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ). It was important to know how this salt interacts with nitrites, but a search of the literature provided little information. According to Lidoff (23), in acid solution hydroxylamine and a gas are formed. Meyer (24) found no reaction whatever to occur in alkaline solution, except in the presence of air when, with  $\text{KNO}_2$ , a small quantity of potassium amido sulfonate crystallized out; in acid solution reduction to nitrous oxide occurs. Gehlen (25) found that alkaline  $\text{Na}_2\text{S}_2\text{O}_4$  reacts with nearly 6 moles of  $\text{NO}$  to yield  $\text{Na}_2\text{SO}_3 \cdot \text{N}_2\text{O}_2$ ,  $\text{Na}_2\text{SO}_4$ ,  $2\text{Na}_2\text{SO}_3 \cdot \text{N}_2\text{O}_2$ , nitrous oxide, and some nitrogen but no products in which hydrogen is directly attached to nitrogen.

*Action of  $\text{Na}_2\text{S}_2\text{O}_4$  on  $\text{NaNO}_2$* —The reaction between these salts is strongly exothermic. The addition of 5 gm. of  $\text{Na}_2\text{S}_2\text{O}_4$  to 1 gm. of  $\text{NaNO}_2$  in 5 cc. of water caused a rise of temperature to  $70^\circ$  in 2 minutes. For the first 2 minutes after mixing, the reaction of the mixture was acid; about the 3rd minute it became neutral and later distinctly alkaline, reaching pH 9 or more. Small bubbles of gas, probably nitrous oxide, often formed and impure sodium sulfite and sulfate crystallized. The mother liquor contained  $\text{NaNO}_2$ ,  $\text{NaOH}$ , and, often for some days, unaltered  $\text{Na}_2\text{S}_2\text{O}_4$ . If the mixture of  $\text{NaNO}_2$  and  $\text{Na}_2\text{S}_2\text{O}_4$  is kept cool during the reaction, the alkalinity at its termination is less; and the reaction is somewhat different if the mixture contains sufficient magnesium sulfate to prevent alkalinity.

That a complex reaction occurs is suggested by the behavior of a mixture of  $\text{NaNO}_2$  and  $\text{Na}_2\text{S}_2\text{O}_4$  with Benedict's solution. This reagent is reduced at room temperature by  $\text{Na}_2\text{S}_2\text{O}_4$  but the addition of a little  $\text{NaNO}_2$  inhibits the reaction. If, however, just sufficient  $\text{NaOH}$  is added to the Benedict's reagent to change the color to ultramarine blue, much larger quantities of  $\text{NaNO}_2$  can be added to  $\text{Na}_2\text{S}_2\text{O}_4$  without destroying its reducing action in the cold.

The formation of nitric oxide by the action of  $\text{Na}_2\text{S}_2\text{O}_4$  on nitrite may be shown by gasometric estimations and by the reaction with ferrous sulfate. When  $\text{NaNO}_2$  is shaken with a dilute mineral acid,  $\text{NO}$  is gradually given off. If a little  $\text{Na}_2\text{S}_2\text{O}_4$  is added to the solution, gas (in this case a mixture of  $\text{NO}$ ,  $\text{N}_2\text{O}$ , and possibly some  $\text{N}_2$ ) is liberated more readily, but with increasing quantities of  $\text{Na}_2\text{S}_2\text{O}_4$  the amount of gas given off gradually diminishes until with approximately equimolecular quantities of  $\text{NaNO}_2$  and  $\text{Na}_2\text{S}_2\text{O}_4$  no gas or only a small quantity of nitrous oxide is liberated by dilute acid.



An increased pressure of nitric oxide can be demonstrated by ferrous sulfate, even in slightly alkaline media. Thus if  $\text{Na}_2\text{S}_2\text{O}_4$  is added to a 1 per cent solution of  $\text{FeSO}_4$  and the mixture made slightly alkaline, the addition of a crystal of sodium nitrite causes a brown coloration before further changes occur. Or the interaction of  $\text{NaNO}_2$  and  $\text{Na}_2\text{S}_2\text{O}_4$  may be followed by adding a dilute ferrous solution to the mixture at intervals. With equal weights of the salts the brown coloration will be found to diminish within 1 minute after mixing and gradually to become less and more delayed. But it is obtained well into alkalinity, although for observation it may be necessary to filter off the ferrous hydroxide formed. Some increased pressure of NO can be demonstrated whether the sodium nitrite or the sodium hyposulfite is in excess.

It is probable that other reducing agents capable of changing oxyhemoglobin to hemoglobin also facilitate the liberation of an increased pressure of NO from  $\text{NaNO}_2$ , since they produce nitric oxide hemoglobin in combination with nitrites. Sulfides, however, are somewhat peculiar and receive separate consideration below.

*Action on Blood*—When a freshly prepared mixture of  $\text{NaNO}_2$  and  $\text{Na}_2\text{S}_2\text{O}_4$  in solution is added to a suitable dilution of blood, the first change is to hemoglobin but in the course of a few minutes the plum color of hemoglobin gradually changes to a rose-red and spectroscopically the single band of hemoglobin is seen to be divided and to be unaffected by the addition of excess of  $\text{Na}_2\text{S}_2\text{O}_4$ . Nitric oxide hemoglobin has been formed. If the two substances are added separately, the final product is the same. When nitrite is added first and methemoglobin is formed, there is primarily reduction to hemoglobin and then the change to NO hemoglobin as before. Since NO hemoglobin is relatively insensitive to variations in pH, the reaction is obtained over a wide range, though not with the same facility. Alkalinity diminishes the pressure of NO formed and consequently slows down the reaction. Even on the acid side of neutrality differences are observed. Brooks (26) found that, "The times for 50% change of reduced haemoglobin to NO-haemoglobin with the experimental conditions used were very roughly 10, 4 and 1 min. at pH 5.66, 5.38 and 5.15 respectively and 15° C."

*Action of Sulfides*—When ammonium sulfide is added to a solution of blood containing sodium nitrite, the first change, as Gamgee ((1) p. 595) observed, from the methemoglobin formed is to oxyhemoglobin, then to hemoglobin which at first can be readily oxidized again by shaking with air. Finally a change to NO hemoglobin occurs, a result Gamgee did not describe. With freshly prepared ammonium sulfide the NO hemoglobin formed seemed to be relatively pure; and it did not seem material what proportions of nitrite and ammonium sulfide were employed. They only

influenced the rate of the reaction and to a slight extent the final concentration of NO hemoglobin. 1 drop of ammonium sulfide induced the changes in several cc. of a solution of blood saturated with  $\text{NaNO}_2$  and excess of ammonium sulfide produced the same changes on blood containing just sufficient nitrite to convert it to methemoglobin. According to Gamgee, Stokes' reagent produces the same changes as ammonium sulfide. Our experiments record distinct differences. The reduction to hemoglobin is more rapid and the previous formation of oxyhemoglobin is less distinct and less easily observed; and on shaking with air, after hemoglobin has been formed, the oxyhemoglobin remains unreduced for a much longer period of time.

The change to oxyhemoglobin is not obtained with  $\text{Na}_2\text{S}_2\text{O}_4$  or other reducing agents; nor is the methemoglobin formed by potassium ferricyanide, quinone, acids, and other agents converted through oxyhemoglobin to hemoglobin by ammonium sulfide or other reducing substances. By the careful addition of small quantities of a reducing agent to a methemoglobin solution two weak bands can usually be produced which almost certainly are due to a small concentration of oxyhemoglobin, the oxygen having been obtained from such extraneous sources as the dissolved air in the methemoglobin solution. Schmidt ((22) p. 228) obtained two weak bands on passing hydrogen through a methemoglobin solution but not if the hydrogen had been passed through an alkaline pyrogallol mixture. The oxyhemoglobin bands in the case of ammonium sulfide reduction of blood containing nitrite are much more intense, a fact which suggests that the necessary oxygen probably comes from the nitrite itself. This view is supported by the reaction of leucomethylene blue to the presence of nitrite in blood. If a crystal of sodium nitrite is added to a solution of blood containing a small quantity of leucomethylene blue (produced by adding the proper amount of  $\text{Na}_2\text{S}_2\text{O}_4$  to a solution of methylene blue), a marked blue color soon develops about the crystal. The action is not connected with any peroxidase activity; cyanides do not interfere with the action. The result indicates that atomic oxygen is available; but, since nitrites also accelerate the reduction of oxyhemoglobin to hemoglobin by ammonium sulfide, the whole system would appear to have become more labile. In the presence of ammonium sulfide experiments with leucomethylene blue proved indecisive, probably owing to secondary actions.

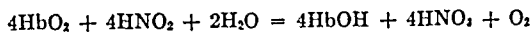
Pure sodium sulfide does not induce the same reactions as ammonium sulfide on nitrated blood. For blood, sodium sulfide is an unsatisfactory reducing agent. In concentrations which produce hemoglobin from oxyhemoglobin it is very slow in action, and somewhat larger concentrations carry the reduction to reduced hemochromogen. The addition of nitrite

slightly accelerates and intensifies the last named change but seems to have no other action. This somewhat puzzling reaction of ammonium sulfide with nitrited blood was not further investigated, but it is interesting to note that it only occurs with ammoniacal reducing agents (ammonium sulfide and Stokes' reagent), and ammonia, as will be shown later, has an inhibiting effect on the action of nitrite on blood. A mixture of ammonia and  $\text{Na}_2\text{S}_2\text{O}_4$ , however, did not produce the sequence of changes.

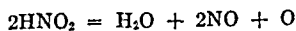
### *Effect of Oxidizing Agents*

It will be sufficient to consider the effect of potassium ferricyanide. Its action on blood differs from that of nitrites ((16) p. 446) and comparison must be limited to the formation of methemoglobin. Points for consideration are the following. (a) Potassium ferricyanide liberates oxygen quantitatively when added to blood. Nitrites do not liberate oxygen. (b) Ammonium sulfide changes nitrite-produced methemoglobin primarily to oxyhemoglobin but does not have this effect on ferricyanide-produced methemoglobin. (c) Ammonia scarcely interferes with the action of ferricyanide on blood, but markedly inhibits the action of nitrites. (d) The reaction of  $\text{H}_2\text{O}_2$  with methemoglobin produced by ferricyanide differs from that with methemoglobin produced by nitrite. (e) The spectroscopic changes which may be observed when a properly apportioned mixture of nitrite and HCN is added to blood are not seen with a mixture of ferricyanide and HCN.

*Gasometric Estimations*—Gamgee's careful analyses showed no absorption of oxygen when nitrites acted on blood ((1) p. 601). Von Zeynek (27) found no oxygen given off. Meier ((18) p. 255) investigating the gaseous changes in acid, neutral, and alkaline solutions of hemoglobin with 1, 10, 100, and 1000 molar quantities of nitrite found that with the higher amounts no oxygen was given off, and that no change occurred in the gas volume when the mixture was neutral or alkaline. In acid mixtures he obtained a slow evolution of oxygen—the reaction could not have taken more than a few minutes—amounting, for a complete change to methemoglobin, to a quarter of the available oxygen of the oxyhemoglobin. From this experiment he suggests that the reaction at pH 5 is

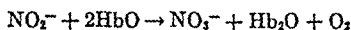


Haurowitz (28) had previously suggested that methemoglobin formed by nitrite was produced by oxidation according to the equation



Recently Greenberg, Lester, and Haggard ((20) p. 670) have stated that in four determinations by the micro gasometric method of Roughton and

Scholander, with no excess of nitrite, a liberation of 0.80, 0.77, 0.79, and 0.80 mole of oxygen per mole of hemoglobin occurred, presumably in neutral solution. Meier (18) had employed 1 mole of  $\text{NaNO}_2$ , having found 0.5 mole insufficient to convert oxyhemoglobin to methemoglobin even at pH 5. For the reaction with 0.5 mole of  $\text{NaNO}_2$  Greenberg, Lester, and Haggard suggested the following equation.



This equation shows the liberation of half the available oxygen of the oxyhemoglobin. In the many estimations we have made with Haldane's apparatus with different kinds of blood at varying pH the changes of gas volume have been slight. When 1.3 gm. of oxyhemoglobin were treated with a little more than an equimolecular quantity of  $\text{NaNO}_2$ , only 0.1 cc. of gas was developed at pH 5.2 and 0.05 cc. of gas at pH 7.2. When saturated solutions of  $\text{NaNO}_2$  were used, some absorption of gas occurred. In two large scale experiments in which the oxygen was estimated by absorption in alkaline pyrogallol no material absorption or evolution of oxygen was found. In one case the oxygen content of the enclosed air was, after 3 hours, 20.9 per cent; in the other it was 20.8 per cent after 5 hours; in both experiments excess of nitrite was used. We, therefore, cannot admit that the equation of Haurowitz or of Meier or of Greenberg, Lester, and Haggard represents the primary action of nitrite on blood.

*Effect of Ammonia on Nitrite Action*—Ammonia has an inhibiting effect on the action of nitrite on blood and in some respects alters the reaction. The effect is well seen by comparing the actions in ammonia and sodium bicarbonate solutions. Usually  $\text{m}/100$   $\text{NaNO}_2$  in 5 per cent  $\text{NaHCO}_3$  solution will induce changes in the oxyhemoglobin of human blood added to it within 1 minute; but  $\text{m}/3$   $\text{NaNO}_2$  in 0.15 per cent ammonium hydroxide dilution causes only slight darkening of the blood after mixing and the oxyhemoglobin bands can be observed for many hours. Eventually some methemoglobin, possibly some NO hemoglobin, and degradation products are formed.  $\text{m}/30$   $\text{NaNO}_2$  in 0.05 per cent ammonium hydroxide dilution produces similar changes.

Blood added to the strongest ammonia solution shows no immediate changes. In 5 minutes traces of hematin can be detected and almost complete conversion to it and to commencing further degradation products occurs within an hour. Methemoglobin is not formed. If strong ammonia solution is diluted with 1 or better more volumes of water prior to adding the blood, alkali methemoglobin and, with relatively weak concentrations such as 5 per cent, some acid methemoglobin occur as a transitory product. Human blood added to the strongest ammonia solution saturated with  $\text{NaNO}_2$  reacts as with ammonia solution alone; and blood, added to 3 per

cent ammonium hydroxide dilution saturated with sodium nitrite, undergoes no obvious immediate change. After several hours there is some general absorption of the spectrum but the oxyhemoglobin bands are still well marked. Gradually the blood pigment is precipitated as hematin.

Ammonia solution does not inhibit the formation of nitric oxide hemoglobin when nitrite is added to a solution of reduced hemoglobin. It even appears to accelerate it slightly.

*Effects of Hydrogen Peroxide*—If a small amount of nitrite is added to a solution of blood and methemoglobin obtained, the addition of a very small quantity of hydrogen peroxide will cause the brownish colored solution to pass through a dirty amethyst color to an olive-green. If the change is watched with a spectroscope, the methemoglobin band in the red region will be found to disappear very quickly, the farther band in the green will be seen to fade more slowly, and, concurrently, there is a strengthening of the band about the D line until it becomes the only band in the spectrum. If the correct amount of hydrogen peroxide for the nitrite and blood has been added, this band may be observed for an hour or more, but generally it dissipates, leaving behind a slight general absorption. If, when the green stage has been reached,  $\text{Na}_2\text{S}_2\text{O}_4$  is added to the solution, the color becomes purer green, and the band about the D line disappears and is replaced by an intense band in the position of the methemoglobin band in the red. Soon two faint bands develop about the position of the oxyhemoglobin bands: They are probably due to the formation of a small concentration of NO hemoglobin. All the bands are fairly permanent and are uninfluenced by further additions of  $\text{Na}_2\text{S}_2\text{O}_4$  or by adding strong ammonia solution; and they will bear NaOH to the production of alkalinity but are destroyed by excess. The changes are obtained if the  $\text{NaNO}_2$  and  $\text{H}_2\text{O}_2$  are mixed prior to being added to the blood. The reaction is not always successful and the optimal conditions for even one kind of blood were not determined. However, if 0.5 cc. of 3 per cent  $\text{H}_2\text{O}_2$  is added to 10 cc. of  $\text{M}/10$   $\text{NaNO}_2$  and 0.5 cc. of the mixture is added to 10 cc. of 2 per cent blood dilution (say of pH 6), the change may usually be observed. The amounts of  $\text{NaNO}_2$  and  $\text{H}_2\text{O}_2$  required depend upon the pH of the solution of blood. It is essential that the quantity of  $\text{NaNO}_2$  added be sufficient to convert the oxyhemoglobin to methemoglobin; the amount of  $\text{H}_2\text{O}_2$  necessary is relatively small. The reaction is more difficult to obtain with increasing pH but there was evidence of it at pH 8 and in a saturated solution of  $\text{NaHCO}_3$ . With these higher pH mixtures stronger reagents, e.g. 6 per cent hydrogen peroxide in  $\text{M}/1$  sodium nitrite, are needed.

The small amount of hydrogen peroxide required and the necessity for conversion of the oxyhemoglobin to methemoglobin suggest that the compound formed is similar to the compounds of peroxide with methemo-

globin described by Keilin and Hartree (29). But the nitrite product differs from these in having only one absorption band, in being more lasting, and in reacting differently with  $\text{Na}_2\text{S}_2\text{O}_4$ . Peroxide methemoglobin prepared from potassium ferricyanide is reconverted to methemoglobin by minute amounts of  $\text{Na}_2\text{S}_2\text{O}_4$  and to hemoglobin by further additions. Methemoglobin solutions produced by quinone or by acids are merely reoxidized by the addition of  $\text{H}_2\text{O}_2$ . And it is interesting that organic nitric esters do not give the reaction, the addition of  $\text{H}_2\text{O}_2$  to a solution of methemoglobin produced by methyl nitrate or by ethylene-glycol dinitrate forming oxyhemoglobin when the peroxide is in sufficient amount; minute quantities seemed to be without action. Nitrite inhibits the catalase action of blood; organic nitrates do not. Keilin and Hartree (29) found that catalase interfered with the formation of peroxide methemoglobin compounds.

*Effect of Cyanide*—Cyanide added to nitrated blood solutions forms cyanmethemoglobin, as cyanide will when added to methemoglobin solutions however produced. But the nitrite reaction is peculiar in that the reaction through methemoglobin to cyanmethemoglobin can be followed spectroscopically, which is not the case with any other methemoglobin-forming substance tried. If blood is added to a properly proportioned mixture of  $\text{NaNO}_2$  and HCN, the methemoglobin band in the red will be seen to appear, in perhaps half a minute, and gradually disappear, leaving the single broad band of cyanmethemoglobin. The sequence may only be observed within a limited range of HCN concentration which in all cases is small. The observations were made over 30 years ago and only two records can now be found. In one experiment 0.1 cc. of 2 per cent HCN was added to 4 cc. of  $\text{M}/80$   $\text{NaNO}_2$  and 1 cc. of 30 per cent ox blood added to the mixture. The red band of methemoglobin appeared in 30 seconds, increased in intensity for about 15 seconds, began to diminish at the 2 minute interval, and had disappeared in 4 minutes. In the second experiment 0.1 cc. of 2 per cent HCN was added to 20 cc. of 30 per cent blood and 1 cc. of the mixture added at once to 4 cc. of  $\text{M}/80$  sodium nitrite. The red band appeared in 30 seconds, rapidly increased for 20 seconds, and then diminished; after 2 minutes the red band had almost disappeared. Minimal quantities of HCN seemed to cause a slight delay in the formation of methemoglobin when compared with a control having the same concentration of nitrite. With increasing concentrations of HCN the duration of the appearance of the red band was more transient and when the concentration was sufficient it failed to appear. That this sequence is not obtained with potassium ferricyanide we believe is due to a difference in the mode of formation of methemoglobin by the two substances and not to a difference in the velocity of the reaction. An interesting change may be observed when the cyanmethemoglobin,

best in slightly alkaline solution, is reduced with  $\text{Na}_2\text{S}_2\text{O}_4$ . Two well marked bands similar to oxyhemoglobin bands but closer together and nearer the violet end of the spectrum appear. They are better defined, denser, and also nearer the violet end than the bands of NO hemoglobin; the solution of the pigment is relatively insusceptible to further additions of  $\text{Na}_2\text{S}_2\text{O}_4$  and to variation in pH. It is, however, unlike NO hemoglobin, gradually changed to hemoglobin by the continued action of  $\text{Na}_2\text{S}_2\text{O}_4$ .

### *Mode of Action*

The action of nitrite on blood is not a simple ion action attributable solely to  $\text{NO}_2^-$ . It is essentially an action of partly undissociated nitrous acid. This is a relatively weak acid and aqueous solutions of its alkali salts are mildly alkaline. The varying activity of nitrite on blood with varying pH described in the section on velocity reactions seems compatible with the probable hydrolysis. Loew (30) and later Bokorny (31) found nitrites poisonous only to vegetable cells and tissues having an acid sap and concluded that nitrous acid was the active factor. In the animal body Loew suggested that the conversion occurred under the influence of the bicarbonate of the blood which in turn is changed to carbonate. Masoin (8) found that the intravenous injection of sodium carbonate or bicarbonate notably diminished the symptoms of nitrite poisoning.

The action of nitrous acid is of two kinds, one associated with the hydrolysis of  $\text{N}_2\text{O}_4$ , which in a reaction with blood results mainly in the production of methemoglobin, and one associated with the action of NO and the production of NO hemoglobin. The first action is inhibited by ammonia; the second is not. The latter action is the simpler and will be considered first. Undissociated nitrous acid exerts a pressure of NO which is perceptible even in alkaline solutions, and it is this pressure, aided by an increased pressure resulting from the hydrolysis, which leads to the production of NO hemoglobin whenever reduced hemoglobin is formed. Reduced hemoglobin has a great affinity for NO and, over a considerable range of pH, combines with it even under very slight pressures of NO. The extent of reduction to hemoglobin is the limiting factor in the production of NO hemoglobin from nitrite alone. With freshly prepared solutions of  $\text{NaNO}_2$  concentrations considerably in excess of those required to produce methemoglobin are necessary to form appreciable amounts of NO hemoglobin. As NO hemoglobin cannot be formed in the presence of readily available oxygen, when oxyhemoglobin is the starting point, conversion to methemoglobin (to exclude such oxygen) is essential. It has been shown that the concentration of nitrite necessary to form NO hemoglobin is the same for oxyhemoglobin and for methemoglobin, so

that the production of NO hemoglobin by the action of nitrite on oxyhemoglobin would seem to be an effect on or through the mediation of methemoglobin. The effect of  $M/100$  sodium nitrite on methemoglobin is seen in Fig. 8. Larger concentrations of nitrite produce a larger fraction of NO hemoglobin (*cf.*  $M/10$   $\text{NaNO}_2$  in the same figure) but methemoglobin cannot be wholly transformed to NO hemoglobin by the action of  $\text{NaNO}_2$ . To this end the aid of a reducing substance is necessary.

When a reducing agent is added to a solution of  $\text{NaNO}_2$ , the system becomes much more mobile and the pressure of NO is increased. The oxyhemoglobin is reduced and conditions become favorable for the formation of NO hemoglobin. When sufficient reducing agent is present to change the oxyhemoglobin to hemoglobin, complete transformation to NO hemoglobin may occur. The pressure of NO need be but small, since more of it will be liberated as it is taken up by the hemoglobin. Brooks ((26) p. 381) has shown that, "In the presence of a reducing agent 1 mol.  $\text{NaNO}_2$  changed completely one equivalent reduced haemoglobin to NO-haemoglobin at pH 5.15-6.63," and "In the absence of both oxygen and a reducing agent 1 mol.  $\text{NaNO}_2$  combined with two equivalents reduced haemoglobin to give one equivalent NO-haemoglobin and one equivalent methaemoglobin."

It seems probable that in the absence of a reducing agent the reaction cannot occur without traces of oxygen being present. In early experiments with nitric oxide it was our custom to collect sufficient of the gas over very dilute NaOH solution for a day's experiments. On one occasion the gas had to be left for some weeks. On analysis the gas then proved to be mainly nitrous oxide. Thus a dissociation,  $2\text{NO} \rightarrow \text{N}_2\text{O} + \text{O}$ , seems to occur. The accident led to some experiments in which definite quantities of oxygen were added to the nitric oxide before it was shaken with a solution of blood, mainly to ascertain the tolerance of blood to the mixture. The effect of the oxygen was solely to form increased quantities of methemoglobin. NO hemoglobin prepared from blood reduced by pumping and subsequent washing with nitrogen shows a sharpening of the bands when small amounts of  $\text{Na}_2\text{S}_2\text{O}_4$  are added to it. This result which is probably due to the presence of traces of methemoglobin in the preparation has also been noticed by Schmidt (22).

The formation of NO hemoglobin is a consecutive action. The primary action of nitrite on blood is the formation of methemoglobin, acid methemoglobin only at pH 6.2 and alkali methemoglobin only at pH 9.2. The action is generally associated with oxidation, but it is not accompanied by the liberation of oxygen. Conant and Fieser (32) seem to regard the first stage in the formation of methemoglobin as a reduction to hemoglobin, but if this were the case in the action of nitrites we should



expect NO hemoglobin to be formed owing to the affinity of hemoglobin for nitric oxide, a pressure of which must be present in the mixture.

Many investigations have been made on the kinetics of nitrous acid, the most comprehensive having been by Abel and Schmid (33) who also give the previous literature. They show that the accepted equation,  $3\text{HNO}_2 = \text{H}^+ + \text{NO}_3^- + 2\text{NO} + \text{H}_2\text{O}$ , occurs in two steps, the first represented by  $4\text{HNO}_2 \rightleftharpoons \text{N}_2\text{O}_4 + 2\text{NO} + 2\text{H}_2\text{O}$  being very rapid. They further find that the kinetics of the decomposition of nitrous acid are solely the kinetics of the reaction between nitrogen tetroxide and water, the rate of decomposition being that of the hydrolysis of nitrogen tetroxide,  $\text{N}_2\text{O}_4 + \text{H}_2\text{O} \rightarrow \text{HNO}_2 + \text{H}^+ + \text{NO}_3^-$ .

The kinetics of the reactions of nitrous acid in the presence of oxyhemoglobin are still more complex but our experiments seem to have followed the course that might have been expected from Abel and Schmid's investigations. The experiments on reaction velocities appear explicable only on the supposition that nitrous acid is the active factor. The primary action cannot be due to the nitric oxide factor and must be ascribed to the nitrogen tetroxide moiety. The induction period suggests that autocatalytic processes are at work, the hydrogen ion acting as positively catalytic and the nitric oxide pressure and hydroxyl ion as negatively catalytic; and it is probable that the oxyhemoglobin, possibly in part by increasing the hydrogen ion concentration, is also a participant in processes occurring in the inductive period.

The second stage in the kinetics of nitrous acid, the production of equilibrium, was found by Abel and Schmid to be a relatively slow process. It is also relatively slow when nitrite is added to a solution of blood on the alkaline side of neutrality. The kinetics of equilibrium involved in the complete production of methemoglobin are not the same as those of the initial period. It has been shown that if the length of the induction period is taken as an indication of activity, even if latent, the quantity of sodium nitrite required to transform the oxyhemoglobin at various pH values can be represented by a geometrical progression and if plotted shows an acute curve with a sharp rise on the acid side of neutrality. If, however, the quantities of sodium nitrite just sufficient to change oxyhemoglobin to methemoglobin independent of time are plotted, the graph tends to be linear. The following varied examples support the point. (a) The limits of concentration of  $\text{NaNO}_2$  for production of determinable changes on blood are for pH 5.2 M/50,000, for pH 8.2 M/10,000, for pH 9.2 M/2000. (b) The absorption coefficient at  $\lambda$  630 m $\mu$  (bands in the yellow-green cannot be used for this purpose) of M/10,000  $\text{NaNO}_2$  on a diluted mother liquor from oxyhemoglobin crystals was for 2 per cent 1.64, for 1 per cent 0.98, for 0.5 per cent 0.42. (c) The absorption coefficient at  $\lambda$  630 m $\mu$  for the action of M/12,000  $\text{NaNO}_2$  on a solution of oxyhemoglobin

(0.5 per cent mother liquor) was at pH 6.2, 0.46; at pH 7.2, 0.24; at pH 8.2, 0.12. (d) The action of the following concentrations of  $\text{NaNO}_2$  on a solution of 0.33 per cent oxyhemoglobin crystals at neutrality showed absorption coefficients at  $\lambda$  630  $\text{m}\mu$  for  $M/16,000$ , 0.26; for  $M/8000$ , 0.47; for  $M/4000$ , 0.60; for  $M/2000$ , 0.74.

When excessive quantities of  $\text{NaNO}_2$  are used, other factors are involved. There are an increased dissociation and hydrolysis and an increased pressure of NO with the formation of some NO hemoglobin. There is also an increase in the production of acid methemoglobin which is of theoretical interest. If 1 mole of  $\text{NaNO}_2$  will wholly convert 1 mole of oxyhemoglobin to methemoglobin, why is it that many moles of nitrite at the same pH will produce more acid methemoglobin? The point is illustrated in Fig. 7. The curves are those of 0.26 per cent oxyhemoglobin crystals with which solution  $M/6000$   $\text{NaNO}_2$  is equimolar. This strength of  $\text{NaNO}_2$  was not used in this experiment but it would certainly have fallen far short of the absorption caused by  $M/1000$   $\text{NaNO}_2$  in the neutrality graph (Fig. 7, 1, B). At pH 6.2 (Fig. 7, 2)  $M/10,000$   $\text{NaNO}_2$  should produce complete change of the oxyhemoglobin; yet it is markedly exceeded in absorption by  $M/1000$   $\text{NaNO}_2$ . These experiments suggest that with increased concentration of nitrite there develops in oxyhemoglobin solution, possibly under the influence of the oxyhemoglobin, an increase in the concentration of the nitrous acid formed, even in buffered solutions, and a consequent increase in hydrolysis of  $\text{N}_2\text{O}_4$ . These and other experiments described seem to us to raise doubts as to the value of any equation purporting to explain the primary action of nitrite on blood. The consecutive action, the formation of NO hemoglobin, may be a bimolecular reaction which may be formulated but the formation of methemoglobin is certainly more complex.

The hydrolyzed nitrous acid acts upon the globin of the hemoglobin molecule but not to any material extent when equimolar quantities or less are employed. It has a somewhat greater effect upon the proteins of whole blood; and, as previously mentioned, it inhibits in large measure catalase activity.

Sodium nitrite has been used as a type of soluble inorganic nitrite. The action of amyl and other organic nitrites has not been mentioned. Owing to their hydrolysis in contact with water, their effect on the blood is solely that of the nitrous acid thus formed; and as their hydrolysis is variable, they did not prove helpful in elucidating the action of nitrites.

#### SUMMARY

The action of nitrite on blood shows an induction period, a reactionary period, and a stationary period.

The action, it is suggested, is due to hydrolyzed nitrous acid, the primary

effect being the formation of methemoglobin which results from the hydrolysis of nitrogen tetroxide. Its action is inhibited by ammonia.

The formation of NO hemoglobin is a consecutive action resulting from the action of nitric oxide on reduced hemoglobin. It is facilitated by the presence of reducing agents which increase the nitric oxide pressure and cause reduction of the oxyhemoglobin or methemoglobin.

Some differences in the reaction of nitrite and of other substances producing methemoglobin are described.

The effect of sodium hydrosulfite on sodium nitrite is considered.

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# THE TIME REQUIRED FOR ESTABLISHMENT OF THE THERMAL STEADY STATE IN THE HILL-BALDES APPARATUS, WITH APPLICATION TO THE MORE RAPID TERMINATION OF OSMOTIC ACTIVITY

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The time for reaching the thermal steady state is ordinarily the factor which limits the rapidity with which a determination of osmotic activity can be completed by means of Baldes' modification (1, 2) of Hill's vapor tension method (3). In practice, a delay of 15 to 30 minutes or more after drop deposition ensues from this source, depending mainly upon the type of solution being measured (4, 5). The purpose of this paper is to present (a) a calculation indicating that a steady state should establish itself in less than 1 minute, (b) the experimental verification of this calculation, and (c) the application of this result to the more rapid determination of osmotic activity over a wide range of concentrations.

In the following account, familiarity will be assumed on the part of the reader with the Hill-Baldes method, excellent descriptions of which are available (1-7).

*Calculation of Time Course of Establishment of Thermal Steady State—*Consider a drop of a given solution on the thermocouple loop initially at the same temperature as the bath and moist chamber, the filter paper lining the latter being wet with water. The rate of change of the temperature of the drop is given by

$$\frac{d(T - T_0)}{dt} = \frac{dH}{dt} \div C \quad (1)$$

in which  $T$  is the absolute temperature of the drop,  $T_0$  is the absolute temperature of the bath and moist chamber,  $dH/dt$  is the algebraic sum of heat gain and heat loss, and  $C$  is the heat capacity of the drop, taken to be equal in calories to the volume of the drop in cc. For the rates of heat gain and heat loss respectively one can substitute the following approximate expressions given by Baldes (2, 4)<sup>1</sup>

<sup>1</sup> All the assumptions and approximations of Baldes (4) are retained. In addition it has been assumed that the rate of change in temperature of the drop is slow compared with the rates at which the diffusion process adjusts itself to conditions in the drop (8).

$$\frac{dH}{dt_{\text{gain}}} = \frac{4\pi LD}{RT_0} \cdot \frac{(P_0 - P)r r_0}{r_0 - r} \quad (2)$$

$$\frac{dH}{dt_{\text{loss}}} = \frac{-4\pi k(T - T_0)r r_0}{r_0 - r} \quad (3)$$

to obtain

$$\frac{d(T - T_0)}{dt} = \frac{\frac{4\pi LD(P_0 - P)r r_0}{RT_0(r_0 - r)} - \frac{4\pi k(T - T_0)r r_0}{r_0 - r}}{\frac{4}{3}\pi r^2} \quad (4)$$

in which  $L$  is the molar latent heat of vaporization,  $D$  is the coefficient of diffusion of water vapor through air,  $P_0$  is the vapor pressure of water at  $T_0$ ,  $P$  is the vapor pressure of the drop at  $T$ ,  $r$  is the radius of the drop,  $r_0$  is the radius of the moist chamber,  $R$  is the universal gas constant, and  $k$  is the thermal conductivity of air.

For  $(P_0 - P)$  one can further substitute  $P_0[\delta - \alpha(T - T_0)]$ , where  $\delta$  is the coefficient of depression of vapor pressure of the drop due to its solutes, and  $\alpha$  is the temperature coefficient of vapor pressure of the drop at  $T_0$ ; and by factoring and simplifying the following is arrived at.

$$\frac{d(T - T_0)}{dt} = \frac{3r_0}{(r_0 - r)r^2 RT_0} [LDP_0\delta - (LDP_0\alpha + kRT_0)(T - T_0)] \quad (5)$$

On integration and suitable manipulation this yields

$$t = \frac{-(r_0 - r)r^2 RT_0}{3r_0(LDP_0\alpha + kRT_0)} \ln \left[ 1 - \frac{\alpha + \frac{kRT_0}{LDP_0}}{\delta} (T - T_0) \right] \quad (6)$$

the constant of integration having been evaluated by assuming that at  $t = 0$ ,  $(T - T_0) = 0$ .

The steady state temperature difference  $(T - T_0)$  at  $t = \infty$  is thus seen to be

$$(T - T_0)_\infty = \frac{\delta}{\alpha + \frac{kRT_0}{LDP_0}} \quad (7)$$

which is identical with the expression for the steady state temperature difference obtained by Baldes by equating the expressions for heat loss and heat gain. If  $x$  is the fraction of the steady state temperature difference attained at any time  $t$ , i.e.  $(T - T_0)/(T - T_0)_\infty$ , then

$$(T - T_0) = x(T - T_0)_\infty = x \cdot \frac{\delta}{\alpha + \frac{kRT_0}{LDP_0}} \quad (8)$$

and expression (6) can be written as

$$t = -A \log_{10} (1 - x) \quad (9)$$

in which  $A$  is a constant which has the value

$$A = \frac{0.766r^2(r_0 - r)}{r_0} \frac{RT_0}{(LDP_c\alpha + kRT_0)} \quad (10)$$

When  $x$  is 0.90 (90 per cent of the steady state attained),  $t$  becomes equal to  $A$ ; when  $x$  is 0.99,  $t$  is equal to  $2A$ ; when  $x$  is 0.999,  $t$  is equal to  $3A$ , etc.

Since the experimental observations to be reported on the rate of steady state attainment were made at  $27^\circ$ , with a chamber of 1.2 cm. radius and a drop of about 0.09 cm. radius, it is of interest to calculate  $A$  for this particular set of conditions. The following values were substituted in expression (10).  $r = 0.09$  cm.;  $r_0 = 1.2$  cm.;  $R = 8.3 \times 10^{-7}$  ergs per degree per mole;  $T_0 = 300$ ;  $L = 1.04 \times 10^4$  calories per mole;  $D = 0.26$  sq. cm. per second;  $P_0 = 3.56 \times 10^4$  dynes per sq. cm.;  $\alpha = 5.9 \times 10^{-2}$ ; and  $k = 5.7 \times 10^{-5}$  calories per second per cc. per degree.  $A$  was found to be 20.2. Therefore, under the conditions in question, by prediction, about 20 seconds should be required for the drop to reach 90 per cent of its steady state temperature, and about 40 seconds to reach 99 per cent of its steady state temperature. At  $20^\circ$ ,  $A$  becomes 27.5, and at  $37.5^\circ$ , it is 13. Drop size also has an important influence. So long as  $r_0$  is large compared with  $r$ ,  $A$  is nearly proportional to  $r^2$ , but relatively independent of  $r_0$ .

*Experimental Determination of Rate of Attainment of Thermal Steady State*—In the usual procedure for determining osmotic activity by means of the Hill-Baldes method, certain disturbing influences operate to prevent rapid establishment of the steady state. The principal disturbing influences are those which arise from the circumstance that, during certain steps in the procedure, the solutions being compared are exposed to thermal conditions different from those prevailing in the thermoregulated moist chamber. These steps are drop deposition, placement of the thermocouple in its moist chamber, and (in the case of some types of chamber) removal of the moist chamber from the water bath. To follow the time course of steady state establishment in the absence of these extraneous factors the special apparatus shown in Fig. 1 and described in the accompanying legend was employed. The water bath was provided with windows so situated as to allow observation of the moist chamber and its contents. The galvanometer in the thermocouple circuit was a Leeds and Northrup type HS with a rated sensitivity of 0.05 microvolt per mm. at 1 meter and a free period of 9 seconds. When necessary, the sensitivity was reduced by an appropriate resistance in parallel.

The following procedure was adopted. The needle and dropping equip-

ment were filled with the solution whose rate of steady state establishment was to be observed. The reference solution (the same as that lining the moist chamber) was deposited in the ordinary way on one thermocouple

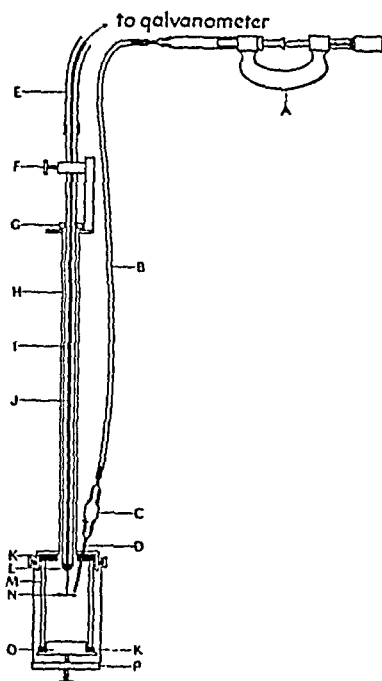


FIG. 1. Schematic sketch of special chamber and dropping apparatus. *A*, micrometer burette; *B*, ureteral catheter; *C*, glass adapter; *D*, hypodermic needle soldered into chamber; *E*, rubber tubing enclosing wires leading from thermocouple to galvanometer; *F*, set-screw permitting adjustment of rotation of thermocouple about its vertical axis; *G*, screw permitting adjustment of height of thermocouple in the chamber; *H*, brass sleeve; *I*, brass tubing to which thermocouple is attached; *J*, thermocouple lead wires; *K*, rubber gaskets; *L*, attachment of thermocouple stem to lead wires; *M*, glass wall of chamber (this is lined with filter paper in which small holes were cut for making the thermocouple visible); *N*, thermocouple reference junction; *O*, brass floor of chamber (also covered with filter paper); *P*, brass brace with screw for sealing chamber.

loop, the other loop under the needle remaining empty. After the solution in the needle had been drawn up away from the needle tip, the thermocouple unit was enclosed in the moist chamber, and the latter immersed in the water bath. Sufficient time was allowed for the moist chamber and its contents to come to temperature equilibrium with the bath. At this junct-

ture the galvanometer was switched into the thermocouple circuit. By manipulations of the micrometer burette, a drop of the solution in the needle was transferred under direct vision to the empty loop, and the time course of the galvanometer deflection recorded. The galvanometer zero remained quite constant during the time of a given observation so that no significant error resulted from failure to employ the reversing switch.

Results for a series of trials with sodium chloride solutions at 27°, and drops with a radius of about 0.09 cm., are shown graphically in Fig. 2. Also

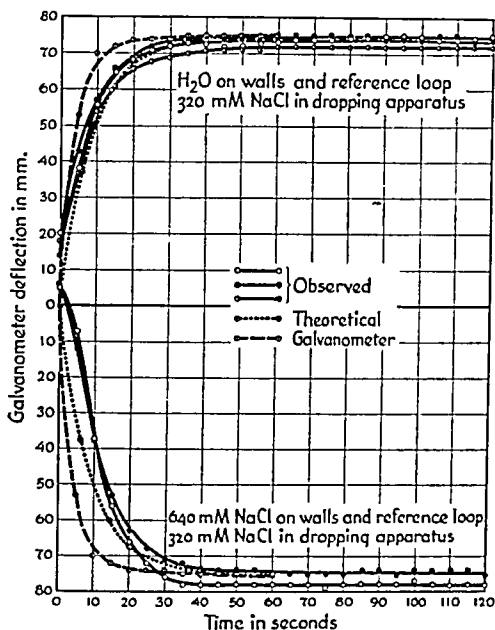


FIG. 2. Rate of attainment of thermal steady state with NaCl solutions at 27°. The special dropping apparatus was used.

included in Fig. 2 for comparison are curves representing theoretical expectations from expression (9), and those for the time course of the galvanometer deflection when the galvanometer was switched into the thermocouple circuit after the steady state had already been established, under very similar circumstances.

It will be noted that the steady state is, indeed, reached under these conditions in approximately 40 seconds.

It will also be observed that the time course of thermal steady state establishment is essentially the same whether the temperature of the drop is increasing (positive galvanometer deflection) or decreasing (negative



ment were filled with the solution whose rate of steady state establishment was to be observed. The reference solution (the same as that lining the moist chamber) was deposited in the ordinary way on one thermocouple

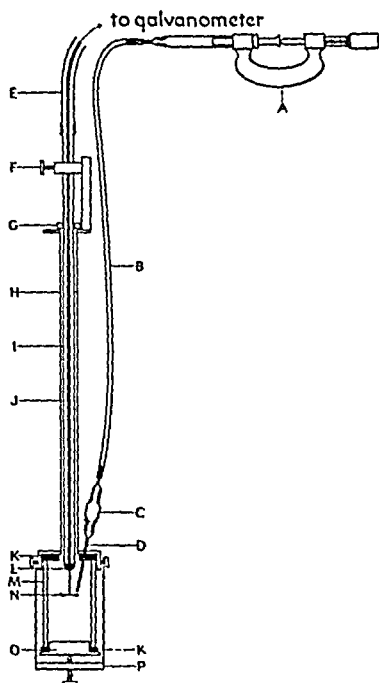


FIG. 1. Schematic sketch of special chamber and dropping apparatus. *A*, micrometer burette; *B*, ureteral catheter; *C*, glass adapter; *D*, hypodermic needle soldered into chamber; *E*, rubber tubing enclosing wires leading from thermocouple to galvanometer; *F*, set-screw permitting adjustment of rotation of thermocouple about its vertical axis; *G*, screw permitting adjustment of height of thermocouple in the chamber; *H*, brass sleeve; *I*, brass tubing to which thermocouple is attached; *J*, thermocouple lead wires; *K*, rubber gaskets; *L*, attachment of thermocouple stem to lead wires; *M*, glass wall of chamber (this is lined with filter paper in which small holes were cut for making the thermocouple visible); *N*, thermocouple reference junction; *O*, brass floor of chamber (also covered with filter paper); *P*, brass brace with screw for sealing chamber.

loop, the other loop under the needle remaining empty. After the solution in the needle had been drawn up away from the needle tip, the thermocouple unit was enclosed in the moist chamber, and the latter immersed in the water bath. Sufficient time was allowed for the moist chamber and its contents to come to temperature equilibrium with the bath. At this junc-

retical ones, it appears reasonable to conclude that the observations lend further support to the validity of Baldes' analysis of the thermoelectric vapor tension method. Moreover, the results, aside from their theoretical implications, justify evaluation of the method as a more rapid means for measuring osmotic activity.

*Rapid Determination of Osmotic Activity over Wide Range of Concentrations*—The manipulation and delays involved in the use of the dropping apparatus preclude its application to a rapid procedure. Instead, a moist chamber which does not require removal from the water bath during drop deposition was employed (see Fig. 3) in connection with the usual manipulating chamber and glass pipettes for drop deposition. The time for

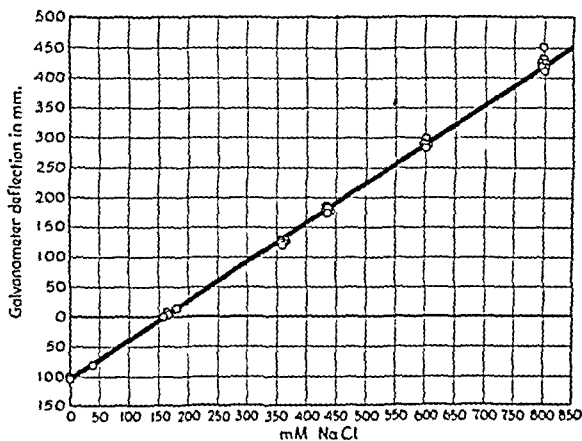


FIG. 4. Galvanometer deflection as a function of concentration for NaCl solutions. The readings were begun about 5 minutes after the drop deposition. Small chamber; bath temperature 37.5°; 157 mM NaCl as standard.

carrying out a determination of osmotic activity by the customary procedure was reduced by (1) shortening the interval between drop deposition and galvanometer reading to one slightly longer than that necessary for steady state establishment, and (2) elimination of drop reversal, correction for asymmetry being made from the calibration deflection on the assumption of a constant asymmetry deflection.

The precision of this "rapid method" may be indicated by the data presented graphically in Fig. 4. Observations were made on sodium chloride solutions up to a concentration of 800 mM (per kilo of water) against a standard of 157 mM at 37.5°. The galvanometer was read approximately 4 minutes after drop deposition, a complete determination requiring about 5 minutes. It was found that the relationship between deflection and con-

centration was essentially linear, and that the average deviation of a single determination from the mean of seven determinations at the 800 mm level was 15 mm. Similar results were obtained when readings were begun 1 minute after drop deposition.

With concentration differences of this magnitude, the rate of change of drop size due to net water transfer may be quite appreciable. For example, under the conditions in question, at the 800 mm level, the decrease in ap-

TABLE I  
*Error of "Rapid Method" for Sweat Samples*

The values are in mm of NaCl per kilo of water.

Sample No.	Osmotic activity		
	Routine procedure	Rapid method	Error of rapid method
1	49	53	4
2	68	65	0
3	68	69	1
4	71	74	3
5	87	89	2
6	88	90	2
7	89	84	-5
8	89	88	-1
9	92	94	2
10	95	94	-1
11	98	98	0
12	98	98	0
13	104	104	0
14	104	103	-1
15	105	103	3
16	117	118	1
17	131	130	-1
18	144	144	0
19	153	154	1
20	159	154	-5
21	171	171	0

parent osmotic activity amounted to 14 mm in 10 minutes. Consequently, regardless of whether or not the "rapid method" is employed, it is necessary under such circumstances to make readings after a standard time interval subsequent to drop deposition.

The accuracy of the "rapid method" may be evaluated by comparing measurements obtained by this method with those obtained by the usual or routine procedure, the latter requiring about 1 hour complete with drop reversal. Such a comparison for samples of human sweat determined at 37.5° in 5 per cent carbon dioxide in oxygen has been made in Table I. It

will be noted that, over a range of 120 mM of sodium chloride, the maximum discrepancy between "rapid method" and routine values was only 5 mM and the average discrepancy about 1.5 mM.

The rapid method is especially suited to the measurement of the osmotic activity of urine, the body fluid which physiologically exhibits the greatest range of variation in solute concentration. The precision and accuracy obtained in determinations on urine appear to be similar to those indicated above.

#### SUMMARY

1. At 27° and with NaCl solutions, the thermal steady state is established in the Hill-Baldes apparatus in approximately 40 seconds. This result is in agreement with a calculation based on Baldes' analysis of the thermoelectric vapor tension method.

2. A procedure is suggested for the use of the Hill-Baldes apparatus as a more rapid method of determining osmotic activity over a wide range of concentrations such as is encountered in sweat and urine.

We are indebted to Mr. William Kubicek and Dr. J. M. Reiner for checking the derivation presented, and to Dr. Austin Henschel and Dr. Henry Taylor for collection of the sweat samples analyzed.

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# DETERMINATION OF DEUTERIUM OXIDE BY MEANS OF THE HILL-BALDES VAPOR TENSION APPARATUS

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At ordinary room and body temperatures the vapor pressure of pure  $D_2O$  is less than 90 per cent of that of  $H_2O$  (1). It is not surprising, therefore, that when  $H_2O$  is placed on one junction of a thermocouple in Baldes' modification (2, 3) of A. V. Hill's vapor tension apparatus (4), and a mixture of  $D_2O$  and  $H_2O$  on the other junction, the galvanometer deflection indicates the  $D_2O$ - $H_2O$  drop to possess a higher temperature, corresponding to a lower vapor pressure, than does the  $H_2O$  drop (5). As the  $D_2O$  leaves the  $D_2O$ - $H_2O$  drop by diffusion to become distributed uniformly in all the water of the thermocouple moist chamber, the process of equilibration can be followed continuously by observation of the galvanometer deflection. The purpose of the present paper is to characterize quantitatively the behavior of  $D_2O$ - $H_2O$  mixtures in the Hill-Baldes apparatus with particular reference to the applicability of the latter to the determination of  $D_2O$  concentration.

## *Methods and Apparatus*

The apparatus employed was similar to that described by Baldes and Johnson (3). The type of moist chamber employed is illustrated in a previous paper ((6) Fig. 3). To deposit drops of a uniform size, a micrometer burette to which was attached a hypodermic needle (gauge 22 to 24) was employed. The bevel of the needle was removed and paraffin or stop-cock grease applied to the outer portion of the tip to prevent creeping of the drop. Time was measured, by stop-watch, from immediately before beginning of exposure of the drop at the tip of the needle. About 10 seconds elapsed until the thermocouple was in place in the moist chamber, the latter being in the water bath continuously. Since, when the galvanometer was switched into the thermocouple circuit, the deflection varied with time, and since a reversing switch was employed to correct for changes in the galvanometer zero position, the procedure for making the galvanometer reading requires description. Simultaneously, at an appropriate time, with noting of the galvanometer reading ( $R_1$ ), the reversing switch was thrown and a second reading ( $R_2$ ) taken when the deflection had attained its maximum in the opposite direction. By means of the reversing switch, the deflection was made to resume its original direction and a third reading ( $R_3$ ) observed.

The interval between  $R_1$  and  $R_3$  was usually 20 seconds. The difference between  $R_2$  and the average of  $R_1$  and  $R_3$  was taken to be the deflection corresponding to the average of the times at which readings  $R_1$  and  $R_3$  respectively were made.

Unless otherwise specified, the observations to be described were made at a bath temperature of  $33^\circ$  with air as the gas phase. Calibration of the deflections for conversion to apparent vapor pressure lowering or apparent osmotic pressure in terms of the molality of an ideal solute (osmolality) was by means of known NaCl solutions, the osmotic coefficient of a 0.1 molal NaCl solution being taken as 0.932 (7).

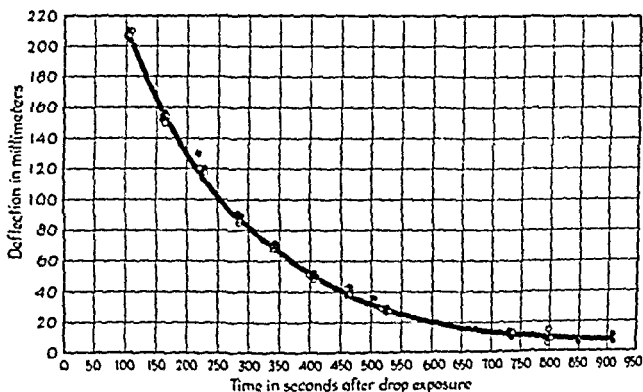


FIG. 1. Typical time-course of deflection curve. 20.14 per cent  $D_2O$  versus  $H_2O$ . Drop size 2.2 c.mm. by burette. Solid circles are readings taken with  $H_2O$  on the walls of the moist chamber; open circles are readings with 20.14 per cent  $D_2O$  on the walls of the moist chamber. Bath temperature  $33^\circ$ .

### Results

*General Observations*—Fig. 1 shows the typical time course of the galvanometer deflection after  $H_2O$  is placed on one, and  $D_2O$ - $H_2O$  on the other, junction of the thermocouple. For some of the trials the filter paper lining the walls of the thermocouple moist chamber was moistened with  $H_2O$ , while in others the walls were moistened with the  $D_2O$ - $H_2O$  mixture. It will be noted that for both sets of conditions the magnitude of the deflection at any given instant was not demonstrably different. The exit of  $D_2O$  from a drop of  $D_2O$ - $H_2O$  placed in an  $H_2O$  environment and the entrance of  $D_2O$  from a  $D_2O$ - $H_2O$  environment into an  $H_2O$  drop appear to have similar time characteristics. In the remaining experiments to be discussed, the walls were moistened with  $H_2O$ .

A linear relation was found to obtain between time after drop deposition and the logarithm of the galvanometer deflection, as illustrated by Fig. 2; that is to say,

$$\frac{dD}{dt} = -kD \quad (1)$$

where  $D$  is the deflection,  $t$  is the time, and  $k$  is a constant. If  $D$  is a measure of  $D_2O$  concentration in the drop, the interpretation of expression (1) is that the rate at which  $D_2O$  escapes from the drop at any given

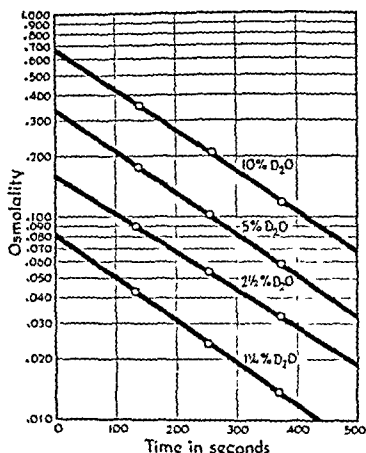


FIG. 2. Relationship between the logarithm of the deflection (expressed as apparent osmotic activity) and time after drop exposure for  $D_2O$ - $H_2O$  mixtures up to 10 per cent  $D_2O$ . Bath temperature  $33^\circ$ . Drop size approximately 2.2 c.mm. For each concentration only one drop was employed and the drop size was only approximately uniform, since deposition was with an ordinary glass pipette (except for the 10 per cent sample).

instant is proportional to its concentration in the drop at that instant.  $k$  then signifies the fraction of the total  $D_2O$  present in the drop which leaves per unit time, and can be evaluated from the slope of lines such as those in Fig. 2. At  $33^\circ$  and a drop size of 2.2 c.mm.,  $k$  is 0.0044 per second. Each 157 seconds, the  $D_2O$  concentration of the drop was reduced by one-half, and complete equilibrium with water in the chamber occurred apparently at the end of about 20 minutes. That the entire drop, and not just an external film or shell, was free from  $D_2O$  was shown by the following procedure. Drops of 99 per cent  $D_2O$  were deposited (with  $H_2O$  on the reference junction) and the deflection followed until it had reached zero, indicating ap-



parent equilibration of the drop  $D_2O$  with the chamber water. When the drop originally containing 99 per cent  $D_2O$  was now removed and re-deposited with a small glass pipette, the deflection still remained at zero. Moreover, the size of the drop had not altered grossly and measurements accurate to about 10 per cent did not demonstrate any such alteration. At  $38^\circ$  and a drop size of 2.2 c.mm., apparent drop  $D_2O$  concentration fell more rapidly than at  $33^\circ$ , halving every 120 seconds. The effect of drop size was only semiquantitatively that anticipated from the change in the relationship between drop surface area and volume, since with decrease in drop size there was observed a disproportionately large increase in rate of  $D_2O$  loss.

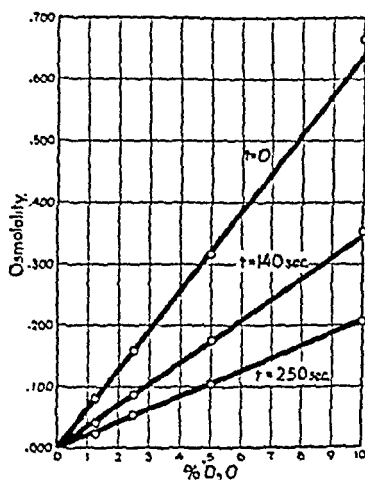


FIG. 3. Relationship between deflection (expressed as apparent osmotic activity) and initial  $D_2O$  concentration at the indicated times after drop exposure, as derived from the data in Fig. 2.

Other factors (drop size, time after drop exposure, bath temperature, moist chamber and its gas phase) remaining constant, the apparent osmotic pressure of  $D_2O$ - $H_2O$  drops appeared to be an essentially linear function of the initial  $D_2O$  concentration, at least up to 10 per cent  $D_2O$ . Such a relationship is shown in Fig. 3 for readings made 0, 140, and 250 seconds after drop exposure. More properly, the apparent osmotic pressure or vapor pressure lowering should have been related to the mole fraction of  $D_2O$  rather than to its per cent by weight; but in the concentration range concerned these two are nearly proportional to one another. The values for zero time, before any  $D_2O$  escape had transpired, were obtained by extrapolation.

The apparent vapor pressure lowering or apparent osmotic pressure at

TABLE I

*Precision of D<sub>2</sub>O Determinations by Hill-Baldes Apparatus*

Samples of the same "group" were examined on the same day with comparable galvanometer sensitivities. The precision for NaCl solutions is included for comparison (concentration of NaCl in molality).

Group	Reference junction	Sample junction	Galvanometer sensitivity	Readings begun at	Galvanometer deflection	Mean	Average deviation	Average deviation $\times 100$ mean	Average deviation in per cent D <sub>2</sub> O
Drops delivered by ordinary glass micro pipette									
A	H <sub>2</sub> O	H <sub>2</sub> O	Low	3	0				
"	"	1.25 % D <sub>2</sub> O	"	3	25, 30, 29, 21, 24, 26	26	2.5	9.6	0.12
"	"	2.50 % D <sub>2</sub> O	"	3	57, 52, 54, 50, 67, 54	56	4.3	7.7	0.19
"	"	5.00 % D <sub>2</sub> O	"	3	116, 115, 103, 126, 107, 100	111	8.0	7.8	0.39
"	"	10.00 % D <sub>2</sub> O	"	3	215, 241, 227, 222, 214, 209	221	8.7	3.9	0.39
B	"	" "	"	3	221, 210, 231, 207, 217, 202	215	8.3	3.9	0.39
C	0.140 NaCl	" "	High	3	83, 81, 113, 58, 50, 54, 140, 117	87	27	3.8*	0.38*
A	H <sub>2</sub> O	0.160 NaCl	Low	3	233, 239, 233, 238, 239	236	3.0	1.3	
Drops of more uniform size delivered by micrometer burette									
D	H <sub>2</sub> O	3.51 % D <sub>2</sub> O	High	2	216, 213, 197, 202, 200, 197, 192, 192, 194, 195, 204, 200, 194, 206, 199, 197, 198, 194, 200, 198	199	4.4	2.2	0.08
				4	123, 124, 117, 118, 116, 115, 111, 111, 114, 116, 124, 118, 114, 119, 115, 114, 115, 112, 114, 116	116	2.9	2.5	0.09
B	"	10 % D <sub>2</sub> O	Low	3	201, 206, 197, 198, 204, 194, 192, 215, 204, 204	200	4.3	2.1	0.21
C	0.140 NaCl	" "	High	3	28, 42, 54, 57, 33, 55, 49, 64, 65, 43	49	10	1.5*	0.15*
"	" "	0.160 NaCl	"	3	93, 91, 92, 94, 90	92	1.5	0.2*	

\* These have been calculated on the basis of the total deflection which would have resulted had H<sub>2</sub>O been used on the reference junction.

zero time per 1 per cent D<sub>2</sub>O was equivalent to an osmolality of  $0.064 \pm 0.003$  ( $\sigma$  distribution), trials separated by several months being included in

this distribution. The equivalent concentration for the 1 to 10 per cent  $D_2O$  range, calculated from the vapor pressure of DHO (1) on the assumption that the vapor pressure of  $D_2O$ - $H_2O$  mixtures is directly proportional to the mole fraction of  $D_2O$ , is approximately 0.066 molal. It may also be added that some observations made with the special dropping apparatus and chamber described elsewhere (6), permitting readings within 65 seconds of, and minimizing disturbances during, drop deposition, yielded a value of 0.065 molal.

*Application to Measurement of  $D_2O$  Concentration*—In view of the above results an attempt was made to evaluate the applicability of the Hill-Baldes apparatus as a method for determining  $D_2O$  concentration. Data on the precision of the method are contained in Table I. With uniform drop de-

TABLE II

*Apparent Osmotic Activity of Solution of NaCl in  $H_2O$ - $D_2O$  Compared with That of NaCl in  $H_2O$  and of  $H_2O$ - $D_2O$  Alone*

The values are given in molality of NaCl.

Time after drop exposure	Apparent osmotic activity			
	0.1008 NaCl in $H_2O$ (a)	Approximately 3 per cent $D_2O$ (b)	Sum of (a) + (b)	0.1008 NaCl in approximately 3 per cent $D_2O$ * (c)
sec.				
130	0.101	0.071	0.172	0.173 (Total)
250	0.101	0.042	0.143	0.142
370	0.102	0.026	0.128	0.126
1500	0.100	0.000	0.100	0.101

\* In this series 0.101 molal NaCl in  $H_2O$  was used as the reference solution.

position by the micrometer burette and a standard (2 to 4 minute) time interval between drop deposition and beginning the galvanometer reading, the average deviation of a single reading from the mean varied from approximately 0.1 per cent  $D_2O$  at the 3.51 per cent level of  $D_2O$  concentration to approximately 0.2 per cent  $D_2O$  at the 10.0 per cent level. This precision is, to be sure, exceeded by the falling drop method (8), but to those who have access to the Hill-Baldes apparatus, the latter affords a rapid means for an approximate  $D_2O$  determination on a minute amount of material. Also, to the extent that the lack of precision is due to the rate at which the deflection changes with time, the precision could be improved by lowering the bath temperature and increasing the drop size, the latter also having the advantage of increasing the accuracy with which relative drop size is known. The maximum precision at present attainable with dilute aqueous solutions, about 0.001 molal, presumably limits that attainable

with  $D_2O$ - $H_2O$  mixtures. At zero time, when the deflection per unit  $D_2O$  concentration is maximum, this corresponds to about 0.015 per cent  $D_2O$ .

The present method has the advantage of permitting, at least under some circumstances, the elimination of the combustion and distillation processes involved in the density methods. Thus, from a series of determinations (see Table II) with 0.101 molal NaCl solutions, it appears that the contributions of NaCl and  $D_2O$  to the apparent osmotic activity are additive, and that the behavior of  $D_2O$  is uninfluenced by the presence of the salt. Consequently, an adequate correction can be made for the NaCl contributions simply by measuring the osmotic activity after the  $D_2O$  has escaped completely from the drop.

#### DISCUSSION

The theory of the steady state condition of the Hill-Baldes vapor pressure apparatus has been given by Baldes (9). This is of particular interest in the study of the effect of a non-volatile solute, such as a salt, on the vapor pressure of water, in which a steady state is rapidly attained.

With a volatile solute the steady state is not attained until the solute has become uniformly dispersed throughout the apparatus. This is the case in the experiments described here on  $D_2O$ - $H_2O$  mixtures.

The complete theory of the apparatus would become rather complex, if it were desired to take into account the full details of the diffusion process and thermal flow in both the liquid and gas phases. Fortunately this does not seem necessary so far as the present experiments are concerned because of two simplifying circumstances: (a) the drop size is quite small, the drop volume being 2.2 c.mm., and the radius consequently about 0.081 cm., and (b) the diffusion coefficient of  $D_2O$  in  $H_2O$  is fairly large at the temperatures used ( $4 \times 10^{-5}$  sq. cm. per second) (10). Due to this the diffusion of the  $D_2O$  molecules within the drop appears to be sufficiently rapid to justify taking the  $D_2O$  concentration as uniform within the drop, at least as a first approximation. The drop may also be assumed to be in quasistatic thermal equilibrium at any instant. The limiting factor determining the process is then the diffusion through the gas phase.

The observed temperature rise of the drop may be considered as a small result of other larger effects. First, there is the compensation between the loss of  $D_2O$  and the gain of  $H_2O$  by the drop. If the whole apparatus were filled with  $H_2O$ , the loss and gain from the drop would exactly balance, but in the present case there is a small excess deposition of  $H_2O$  serving as a source of heat. Secondly, the added heat leads to compensating effects, as is discussed by Baldes (9). Most important of these is the increase in vapor pressure due to the increased drop temperature, and the transfer of heat away from the drop by thermal conduction in the gas phase. It is

interesting to observe that in the attainment of the 'steady state condition of these compensating effects the first (*i.e.*, increased vapor pressure) is the dominant factor. If the gas phase were a thermal insulator, then in the example discussed by Baldes the equilibrium drop temperature would be raised from  $0.067^\circ$  to  $0.09^\circ$ , an increase of 42 per cent. The most important effect of the rise of drop temperature is therefore to tend to annul the inward diffusion of  $H_2O$  by raising the vapor pressure at the drop surface.

The significance of these statements is to bring out the fact that even in the transient condition, which is present in these experiments, the rate of change in the temperature of the drop is slow compared with the rate at which the diffusion process adjusts itself to the conditions at the drop; *i.e.*, to the drop temperature and  $D_2O$  content. If this was not the case, then the instantaneous drop temperature could not be related in any simple way to concentration, nor would it be feasible to apply the *steady state* formula to the non-steady state as has been done by Lifson and Lorber (6).

On the basis of this procedure, it is not difficult to obtain an estimate of the rate of  $D_2O$  loss from the drop for comparison with the observations described above. On the assumption of spherical symmetry for the drop, the diffusion equation in the gas phase is

$$\frac{D\partial^2 Nr}{\partial r^2} = \frac{\partial Nr}{\partial t} \quad r_1 \leq r \leq r_0 \quad (2)$$

$r_1$  being the radius of the drop,  $r_0$  that of the outer container, and  $N$  the concentration of  $D_2O$ .  $D$  is the diffusion coefficient of  $D_2O$  in the gas phase.

We must also consider certain boundary conditions at the surface of the outer container, as well as at the surface of the drop. For the former we use  $N = 0$  at  $r = r_0$ .

For the latter a more indirect argument must be used. Considering the surface of the drop as an energy barrier, the usual arguments of kinetic theory suggest that we take  $N_- = \gamma N_+$  where  $N_-$  is the concentration of  $D_2O$  *inside* the drop, and  $N_+ = N(r_1)$  is that just *outside* the drop. The factor  $\gamma$  can be estimated from the vapor pressure of pure  $D_2O$ .

We therefore set down the boundary conditions at  $r = r_1$ .

$$N_- = \gamma N_+ \quad (3)$$

$$\frac{r_1}{3} \frac{dN_-}{dt} = D \left( \frac{\partial N}{\partial r} \right)_{r=r_1} \quad (4)$$

Equation (4) expresses the continuity of the flow of  $D_2O$  across the surface of the drop.

A certain reduction in equations (3) and (4) can be made by writing

$$\frac{dN_-}{dt} = \gamma \left( \frac{\partial N}{\partial t} \right)_{r=r_1} = \frac{3D}{r_1} \left( \frac{\partial N}{\partial r} \right)_{r=r_1} \quad (5)$$

Combining this with the diffusion equation we get

$$\left(\frac{\partial N}{\partial t}\right)_{r=r_1} = \frac{D}{r_1} \left(\frac{\partial^2 N r}{\partial r^2}\right)_{r=r_1} = \frac{3D}{r_1 \gamma} \left(\frac{\partial N}{\partial r}\right)_{r=r_1} \quad (6)$$

from which

$$\left[\frac{\partial}{\partial r} \left(\frac{\partial N r}{\partial r} - \frac{3N}{\gamma}\right)\right]_{r=r_1} = 0 \quad (7)$$

Let us now try a solution of the general form

$$N(r, t) = v(r) e^{-kt} \quad (8)$$

A solution for  $v(r)$  which fits the boundary condition at  $r = r_0$  is

$$v = A \frac{\sin \left[ \sqrt{\frac{k}{D}} (r_0 - r) \right]}{r} \quad (9)$$

where  $A = \text{constant}$ .

To fit the boundary condition at the surface of the drop we have

$$\begin{aligned} -\gamma \frac{k}{D} \sin \left[ \sqrt{\frac{k}{D}} (r_0 - r_1) \right] + \frac{3 \sin \left[ \sqrt{\frac{k}{D}} (r_0 - r_1) \right]}{r_1^2} \\ + \frac{3 \sqrt{\frac{k}{D}} \cos \left[ \sqrt{\frac{k}{D}} (r_0 - r_1) \right]}{r_1} = 0 \end{aligned} \quad (10)$$

which reduces to

$$x^2 - \left[ 1 + \left( \frac{3}{\gamma} \right) x \cot x \beta \right] = 0 \quad (11)$$

$$x = r_1 \sqrt{\frac{\gamma k}{3D}} \quad \beta = \sqrt{\frac{3}{\gamma}} \left( \frac{r_0}{r_1} - 1 \right) \quad (12)$$

From this equation we are to find the values of  $k$  corresponding to the special solutions of our problem. The final solution is then a linear sum of special solutions with coefficients adjusted to fit the initial conditions. These initial conditions at  $t = 0$  are such that

$$\begin{aligned} N(r) &= 0 \text{ for } r_1 < r \leq r_0 \\ &= \frac{1}{\gamma} N_-^0 \text{ for } r = r_1 \end{aligned} \quad (13)$$

where  $N_-^0$  is the initial concentration of  $D_2O$  molecules in the drop.

We need not carry through the complete determination of this solution if we limit ourselves to a study of conditions after the diffusion process is established in the gas phase; *i.e.*, if we neglect the behavior within the first few seconds after the experiment is started. The leading term of the series has a time dependence given by  $e^{-k_1 t}$  where  $k_1$  is the lowest positive root of equation (11).

The general solution of the transcendental equation (11) is not easy. But if we take account of the numerical values appropriate to this problem we have  $1/\gamma \cong 1.66 \times 10^{-5}$ ,  $\beta \cong 0.105$ , so that the equation becomes in numerical form

$$x^2 - [1 + 0.007x \cot 0.105x] = 0 \quad (14)$$

The positive roots of this equation are very nearly 1, 30, 60 . . . , of which only the first is of interest to us.

TABLE III

Values of  $k$  and  $T$  ("Half Life Time") for  $D_2O$  in  $H_2O$ , from  $k = 3D/\gamma r_1^2$ , at Various Temperatures

$D$  was taken as 0.25 sq. cm. per second and  $r_1$  as 0.081 cm.

Temperature	$\frac{1}{\gamma}$	$k$	$T$
°C.			sec.
20	$1.48 \times 10^{-5}$	$1.37 \times 10^{-3}$	505
30	2.63	2.44	284
40	4.48	4.15	167
50	7.37	6.83	102

The lowest root for  $k$  thus is closely

$$k = \frac{3D}{\gamma r_1^2} \quad (15)$$

We can estimate  $\gamma$  numerically from the expression

$$\frac{1}{\gamma} = \frac{\text{density of saturated } D_2O \text{ vapor}}{\text{density of liquid } D_2O} \quad (16)$$

With the approximate values  $D = 0.25$  sq. cm. per second, and  $r = 0.081$  n., the value of  $k$  has been computed from formula (15). In this way we get the values found in Table III for  $k$ , and the corresponding "half life time" as a function of temperature.

These are to be compared with the measured values of  $k$  of 0.0044 at 33° and 0.005 at 38°, corresponding to half life times of 157 and 120 seconds respectively. Moreover, the formula predicts a mean drop in  $T_0$  of 11.7 seconds per degree between 30–40°, while the measurements give 7.4 seconds per degree between 33–38°. This is considered to be very satisfactory

agreement and it serves to substantiate the interpretation that the galvanometer deflection at any given time actually is a measure of the instantaneous  $D_2O$  concentration.

It may be also pointed out that  $D_2O$  is a special case of a volatile solute, and it may be reasonably expected that the general considerations which obtain for it should hold for other volatile solutes as well.

#### SUMMARY

The behavior of drops of  $D_2O$ - $H_2O$  in the Hill-Baldes apparatus has been characterized quantitatively.

The relationship between the logarithm of the galvanometer deflection and time after drop exposure was observed to be linear. Extrapolation of this relationship to zero time yielded a value for the vapor pressure lowering (apparent osmotic pressure) of a 1 per cent  $D_2O$  solution corresponding to that of a 0.064 molal solution of an ideal solute. In the chamber employed the deflection was reduced by one-half, for each 157 seconds, when the bath temperature was  $33^\circ$  and the drop size was 2.2 c.mm.

The relationship between initial  $D_2O$  concentration and deflection at a given time after drop exposure appeared to be linear up to 10 per cent of  $D_2O$ .

The precision of the apparatus in the determination of  $D_2O$  in  $D_2O$ - $H_2O$  mixtures is such that the average deviation of a single reading from the mean is about 0.1 per cent  $D_2O$  at the 3.51 per cent  $D_2O$  level, and about 0.2 per cent  $D_2O$  at the 10 per cent  $D_2O$  level.

The measured osmotic activity of  $NaCl$  and apparent osmotic activity of  $D_2O$  seemed to be additive.

There is a satisfactory agreement between (1) the time rate of loss of  $D_2O$  from the drop as calculated from theoretical considerations and (2) the observed time rate of decline of the deflection. This, together with certain other observations, supports the interpretation that the deflection at any given time is a measure of the  $D_2O$  concentration in the drop at that time.

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# THE EFFECT OF INGESTED CHOLINE ON THE TURNOVER OF PLASMA PHOSPHOLIPIDS\*

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By the use of labeled phosphorus ( $P^{32}$ ) it was shown that ingested choline speeds up the rate of phospholipid turnover in the liver (1). Betaine had a similar action, but its effect was less pronounced than that of choline (2). As a result of these and other findings (3, 4) it was pointed out in 1939 that phospholipid synthesis by the liver is an important intermediary step in the removal and deposition of liver fat. This view is in keeping with the more recent observations of Boxer and Stetten (5), who have shown with the aid of isotopic ( $N^{15}$ ) choline that the rate of incorporation of *new* choline into phospholipids of the body is retarded during choline deprivation.

The present investigation deals with another phase of the action of choline on phospholipid turnover as measured by the over-all reaction involving the conversion of inorganic phosphate to phospholipid. The additional amounts of radiophospholipids formed under the influence of choline do not long remain in the liver; in the rat they make their appearance in the liver as early as 3 hours after a single feeding of choline but are no longer there 10 hours later (1). The fate of these *extra* amounts of radiophospholipid formed under the influence of choline is considered here

## EXPERIMENTAL

For various periods before the injection of radioactive phosphorus or choline, dogs weighing 10 to 15 kilos were fed a high fat, low protein diet consisting of 38 per cent lard, 8 per cent casein, 44 per cent sucrose, 2 per cent sardilene,<sup>1</sup> 3 per cent brewers' yeast,<sup>1</sup> and 5 per cent Cowgill's salt mixture (7). Each dog received 10 gm. of this mixture per kilo per day, and, unless otherwise noted, this amount was fed once daily at 12.00 noon.

Choline and radioactive phosphorus were administered by stomach tube; at this time the dogs were in the postabsorptive state, having ingested their last meal approximately 18 hours before. As noted below, each dog received radioactive phosphorus on two occasions separated by periods of 9 to 10 weeks; at one of these times choline was fed 30 minutes before the  $P^{32}$ . Each dog received 300 mg. of choline chloride per kilo of body weight

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<sup>1</sup> The vitamin contents of the preparations have been recorded elsewhere (6).

dissolved in 50 cc. of distilled water, whereas labeled phosphorus was administered as 10 cc. of an isotonic solution of  $\text{Na}_2\text{HPO}_4$  containing approximately 0.5 millicurie of  $\text{P}^{32}$ . During the 100 hours after the administration of the labeled phosphate, seven to nine samples of blood were removed from the femoral artery. Food was withheld on the day that  $\text{P}^{32}$  was administered, but feeding was resumed on the following day.

*Methods of Analysis*—The  $\text{P}^{32}$  and  $\text{P}^{31}$  content of the trichloroacetic acid-insoluble fraction of plasma was determined as follows: 1 cc. of plasma was transferred dropwise to a 15 cc. graduated centrifuge tube containing 10 cc. of 10 per cent trichloroacetic acid and the mixture vigorously agitated with a glass rod. It was allowed to stand for 10 minutes and during this time was thoroughly agitated several times to bring the precipitate to a finely suspended state. The mixture was then centrifuged for 5 minutes and the supernatant fluid decanted. The precipitate was washed twice with 5 cc. portions of trichloroacetic acid and centrifuged after each washing. To the precipitate was added 1 cc. of 10 per cent  $\text{NaOH}$ . The mixture was agitated for a few moments with a glass rod and then approximately 3 cc. of water added. For complete solution of the precipitate it was sometimes necessary to place the tube in warm water for a few moments. The final volume in the tube was adjusted by the addition of water to a volume of 4 to 15 cc., depending upon the estimated  $\text{P}^{32}$  activity. The solution was stirred thoroughly and the rod removed, as much as possible of the excess liquid on the rod being transferred to the sides of the tube. After 10 minutes the volume in the centrifuge tube was carefully read and the aliquots for analyses taken immediately thereafter.

For the determination of the  $\text{P}^{32}$  content, 1 to 2 cc. aliquots were transferred to blotters and the radioactivity determined with the Geiger counter. The  $\text{P}^{31}$  content of another aliquot was measured by King's method (8), the color being determined by the Klett-Summerson photoelectric colorimeter.

The petroleum ether-soluble phosphorus of plasma was determined as follows: plasma was first extracted with a 3:1 alcohol-ether mixture, the extract concentrated to a low volume, and the concentrate extracted with petroleum ether. The details of this extraction procedure have been described by Taurog *et al.* (9).

In an earlier communication it was shown that good agreement is obtained between the values for the  $\text{P}^{32}$  content of the trichloroacetic acid precipitate prepared from plasma and the  $\text{P}^{32}$  content of the petroleum ether extract of plasma (10). The data presented in Table I show a similar agreement for the same two fractions so far as  $\text{P}^{31}$  is concerned. In the experiments described here it was necessary to remove as many as nine samples of blood from each dog during a short interval. Hence, in order

to avoid the removal of excessive amounts of blood the  $P^{31}$  and  $P^{32}$  contents of the trichloroacetic acid precipitate of plasma were taken as equivalent to its phospholipid  $P^{31}$  and  $P^{32}$  contents respectively. By the use of this precipitate it was possible to determine the phospholipid  $P^{31}$  and  $P^{32}$  contents in as little as 1 cc. of plasma.

### Results

Phospholipid  $P^{31}$  and phospholipid  $P^{32}$  (radioactive phospholipid) were measured in the plasma of six dogs at various intervals during the 100 hours following enteral administration of 300 mg. of choline chloride per kilo. The results obtained in this type of experiment have been compared with an earlier or later experiment in which  $P^{32}$ , but no choline, was administered

TABLE I

*Comparison of Petroleum Ether-Soluble Phosphorus of Plasma with Plasma Phosphorus Insoluble in Trichloroacetic Acid*

Plasma sample No.*	1	2	3	4	5	6	7	8	9
Petroleum ether-soluble†	14.6	15.7	16.6	10.5	11.4	17.2	17.0	11.2	11.4
Trichloroacetic acid-insoluble	14.7	16.0	16.3	10.7	11.8	17.7	17.9	11.8	11.4

\* Sample 1 was obtained from a dog 14 hours after the ingestion of a diet high in lean meat. The other plasma samples were obtained from two dogs that had been fed the high fat, low protein diet described in the text; Samples 2 to 5 were taken 48 hours after the ingestion of the last meal, whereas Samples 6 to 9 were obtained 9 hours after the ingestion of food.

† Plasma was first extracted with alcohol and ether, concentrated to a low volume, and the concentrate extracted with petroleum ether. The details of this procedure are described elsewhere (9).

to the same dog. In this way each dog served as its own control in demonstrating the effects of choline on the turnover of plasma phospholipids.

*Effect of Single Feeding of Choline on "Specific Activity-Time Curve" of Plasma Phospholipid P*—The specific activities shown for Dogs 26A, 26B, 36A, and 36B in Table II and for Dogs 2A and 2B in Figs. 1 and 2 respectively represent the ratio of percentages of injected  $P^{32}$  recovered as phospholipid per 100 cc. of plasma to mg. of phospholipid  $P^{31}$  per 100 cc. of plasma. The results recorded demonstrate the rapidity with which radiophospholipids appear in the plasma. Thus in the 4 hours between the 2nd and 6th hour after the injection of the radioactive phosphorus, the specific activities of plasma phospholipid phosphorus increased about 10-fold. Maximum values for specific activities were found at different intervals in the six dogs studied; they were observed as early as 24 hours and as late as 36 hours after the injection of the radiophosphorus. The last measurement

TABLE II

*Effect of Ingested Choline on Specific Activity of Plasma Phospholipid Phosphorus*

Dog 26A*			Dog 26B*			Dog 36A†			Dog 36B†		
Interval after $P^{32}$ administration‡	Specific activity of phospholipid P		Interval after $P^{32}$ administration‡	Specific activity of phospholipid P		Interval after $P^{32}$ administration‡	Specific activity of phospholipid P		Interval after $P^{32}$ administration‡	Specific activity of phospholipid P	
	Control	Choline		Control	Choline		Control	Choline		Control	Choline
hrs.			hrs.			hrs.			hrs.		
2	0.36	0.45	2	0.26		2	0.23	1.28	2	0.15	0.38
6	3.71	4.99	6	4.43	4.82	6	3.50	8.81	6	3.42	4.75
12	12.0	13.2	12	10.2	9.64	10.5	9.65		10.5	8.24	
18		16.8	18	13.3		12		18.7	12		13.9
24	15.6		24		17.5	18	14.3	24.2	18	10.3	21.0
27		22.0	27	16.4		24	15.9	28.3	24	10.2	21.2
36	15.7	20.5	36	16.8	15.1	32		28.7	33	14.3	23.4
50	15.2		50		15.3	34	18.0		38		23.9
54		16.6	54	16.9		38		28.2	51	11.0	20.6
100	10.1	10.4	100	14.2	10.9	50	15.8		101	10.5	13.9
						52		21.4			
						101	10.7	13.1			

\* The control and choline experiments were separated by an interval of 10 weeks.

† The control and choline experiments were separated by an interval of 9 weeks.

‡ Choline was administered 30 minutes before the radioactive phosphorus.

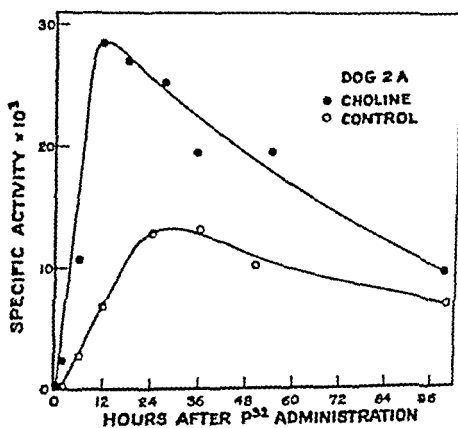


FIG. 1. The effect of choline on the "specific activity-time relations" of plasma phospholipid phosphorus in Dog 2A.

was made at 100 hours; at this interval the values for specific activities were considerably below the maximum but still above the values found at the 6 hour interval.

to phospholipid in the kidney and small intestine (11); yet negligible amounts of radiophospholipid were recovered in plasma (11). It seems unlikely, therefore, that phospholipids synthesized by kidney or small intestine could account for the striking effects of choline on the specific activities of plasma phospholipid phosphorus.

Practically all phospholipids of the plasma are of the choline-containing type (9). In view of the close relationship that exists between liver and plasma (11), there can be little doubt that the ingestion of choline increases the removal of newly formed choline-containing phospholipids from the liver. Whether or not choline affects the non-choline-containing phospholipids of the liver remains to be determined.

The mechanism by which choline increases the specific activity of plasma phospholipid phosphorus is not known at present. Since this occurs in the absence of a change in the total phospholipid content of the plasma, it would appear that choline increases the utilization of phospholipids. Such an increase in the utilization of phospholipids could mean either (1) an increased transport of phospholipid from liver to peripheral tissues or (2) an increased breakdown of choline-containing phospholipids within the liver itself. In either case the increased specific activity of plasma phospholipid phosphorus is merely a reflection of increased specific activity of the choline-containing phospholipids of the liver.

Diet is of importance in eliciting this stimulating effect of choline upon phospholipid renewal. It was observed here in dogs fed a low protein, high fat diet but not in dogs fed a diet high in lean meat. The failure to obtain an acceleration of phospholipid turnover by the administration of extra choline in the dogs fed a diet high in lean meat may be due to the fact that the food ingested already contained sufficient choline (and probably other factors) to bring about maximum stimulation of phospholipid turnover.

#### SUMMARY

1. The effect of choline on the renewal of plasma phospholipid phosphorus was studied in dogs fed a high fat, low protein diet. Radioactive phosphorus was used as the labeling agent.

2. A single feeding of 300 mg. of choline chloride per kilo of body weight accelerated phospholipid turnover in plasma.

Choline increased the rate of change in the specific activity of phospholipid phosphorus of plasma during the early intervals after the administration of radioactive phosphorus.

The maximum values found for the specific activity of plasma phospholipid phosphorus were higher in dogs fed choline than in dogs fed no choline.

administration; maximum effect was observed in about 24 to 36 hours; its effect was no longer detectable in 100 hours.

*Effect of Single Feeding of Choline on Total Phospholipid Content of Plasma*—The influence of a single ingestion of 300 mg. of choline chloride per kilo of body weight upon the phospholipid content of plasma was determined in eight dogs. The treatment of six of them (Dogs 2A, 2B, 26A, 26B, 36A, and 36B) has been described above; determinations of the total phospholipid content of their plasma were made in order to obtain the values for specific activity recorded in Table II.

Dogs A and B were fed the high fat, low protein diet for 14 days. At 8.00 a.m. of the 15th day, 300 mg. of choline chloride per kilo were administered to each dog by stomach tube and the phospholipid P of their plasma determined at various intervals during the next 50 hours. Food was withheld on the day that the choline was administered, but feeding was resumed the next day. In other words, during the 50 hours of observation, Dogs A and B received a single feeding of the high fat, low protein diet 27 hours after the administration of the choline.

To serve as a control for this choline experiment, the phospholipid P of the plasma of Dogs A and B was measured 3 days earlier, at which time no choline was administered. Care was taken to maintain all conditions the same in both the control and the choline experiments with respect to the feeding of the dogs and the times when blood samples were removed.

In none of the eight dogs examined was a measurable change in the phospholipid level of plasma produced by the single feeding of 300 mg. per kilo of choline chloride. This was particularly well brought out in Dogs A and B, in which the interval between the control and choline experiments was quite short; namely, 3 days.

#### DISCUSSION

It was demonstrated recently in this laboratory that plasma phospholipids are synthesized mainly in the liver (11) and hence there is good reason to believe that the liver is responsible for the increased specific activities of plasma phospholipid phosphorus that are shown here to result from the ingestion of choline. The view that the primary action of choline is on the liver and that the increased phospholipid activity of plasma merely reflects such accelerated activity in the liver receives further support from the following evidence: surviving slices of liver (12), kidney (13), brain (14), muscle,<sup>3</sup> and small intestine,<sup>3</sup> but not plasma,<sup>3</sup> have been shown to be capable of incorporating inorganic phosphate into phospholipid molecules. Again, when radioactive phosphate was injected into the liverless dog, it was found that in 6 hours significant amounts of the labeled phosphate were converted

<sup>3</sup> Unpublished observations.

# MICRODETERMINATION OF GLYCOCYAMINE AND ARGININE BY MEANS OF A SYNTHETIC ION EXCHANGE RESIN FOR CHROMATOGRAPHIC SEPARATION

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Considerable evidence has accumulated to support the view that glyco-  
cyamine (guanidoacetic acid) is a normal precursor of creatine in the  
animal body (1-3). Hence, a simple reproducible method for the measure-  
ment of glyco-  
cyamine would prove a valuable tool for the clinical study of  
the transmethylation involved in this conversion. The abundance in the  
literature of modifications of Weber's (4) original method is evidence of  
the difficulty of reproducing analytical results by this procedure. All  
the earlier adaptations included suitable adsorption and elution of the  
glyco-  
cyamine together with the arginine, followed by repeated batch  
adsorption of the arginine on permutit. Glyco-  
cyamine and arginine are  
the only common biological substances that yield a color in the Sakaguchi  
reaction. The maximum tolerated amounts of potential interfering sub-  
stances have been determined by Dubnoff (5). One of the difficulties in  
reproducing results by various methods appears to have been the unpre-  
dictable variations in different lots of permutit. A second drawback was  
that the earlier procedures developed by Luck (6) required the determi-  
nation of the optimal amount of hypobromite necessary for color develop-  
ment for each unknown solution. Dubnoff and Borsook (7), who have  
published the most recent modification of the method, by adsorbing the  
arginine on a simple permutit column, obtained excellent separation, at  
the same time avoiding the laborious steps of earlier procedures. They  
gained a high degree of stability of color in the Sakaguchi reaction and a  
greater resistance to interfering substances by the device of adding the  
urea before the hypobromite. Nevertheless, again various laboratories  
have been unable to reproduce readily the results originally obtained.<sup>1</sup>

In this laboratory assorted lots of permutit gave various results, all  
unsatisfactory. For this reason an ion exchange substance was sought  
which would be sufficiently uniform to give reproducible results. Such  
a reagent was found in the formaldehyde-phenolic condensation product,

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Medicine.

<sup>1</sup> Personal communications.



Amberlite IR-100-H.<sup>2</sup> The operating characteristics of this material have been described by Myers *et al.* (8). The development of color in the procedure described follows quite closely that used by Dubnoff, with minor modifications to yield a lighter blank. The relationship of concentration of glycoeyamine to intensity of color was found to be curvilinear, rather than rectilinear as it had been originally described.<sup>3</sup> The more sensitive method of developing color by successive additions of urea and hypobromite described by Macpherson (9) was not employed. An apparatus to control the speed of down flow through a battery of small columns has been devised.

With suitable elution, according to the directions of Dubnoff (5), the method should be equally applicable to the determination of arginine.

#### *Reagents—*

Sodium chloride solution, 0.3 per cent.

Amberlite IR-100-H, prepared as described below.

$\alpha$ -Naphthol, 0.2 per cent stock solution in absolute alcohol. The  $\alpha$ -naphthol should be repeatedly recrystallized until, when dissolved in a minimal volume of alcohol, it yields a colorless fluid. Crude commercial  $\alpha$ -naphthol can be best purified by solution in heated alcohol, followed by the addition of hot water until supersaturation is approached. The supernatant fluid is then drawn off and cooled, while the amber residue containing impurities is again extracted in a similar manner.

Urea, 16 per cent solution.

$\alpha$ -Naphthol-urea reagent. Immediately before use the  $\alpha$ -naphthol, which should be made up daily, is added to the urea solution in the proportions of 0.2 ml. of  $\alpha$ -naphthol to 1.5 ml. of urea.

Sodium hypobromite solution. 0.66 ml. of liquid bromine added to 100 ml. of 5 per cent sodium hydroxide. This remains stable in the refrigerator as long as 4 months.

#### *Procedure*

Urine for the analyses reported was diluted 1:6 with water. It would probably be advantageous to vary the dilution according to the specific gravity of the urine in a manner similar to that recommended by Tierney and Peters (10) for the determination of urine creatine. The method has been designed for application to ultrafiltrates of serum prepared as prescribed by Danowski (11). These should be diluted with 2 parts of water to reduce the salt concentration to approximately 0.3 per cent.

<sup>2</sup> The author is indebted to the Resinous Products and Chemical Company, Washington Square, Philadelphia, for supplying the resins used.

<sup>3</sup> Under the conditions defined by Dubnoff the break in linearity is barely perceptible, but definite. Murray and Luck mention that two colors must be involved in the Sakaguchi reaction.

The arginine and glycoeyamine are adsorbed on a column of Amberlite IR-100-Na, from which the glycoeyamine is subsequently eluted with 0.3 per cent sodium chloride solution. The analytical grade of the resin, IR-100-H, should be employed. It is essential that this be converted entirely to the sodium form. This is effected by treating each gm. of resin in a suitable column with 10 ml. of 5 per cent sodium chloride solution, allowing at least 10 hours total down flow time. The column is then washed with distilled water, after which the resin is stored in the refrigerator in 0.3 per cent sodium choride solution. Thus stored it remains efficient for at least 6 months.

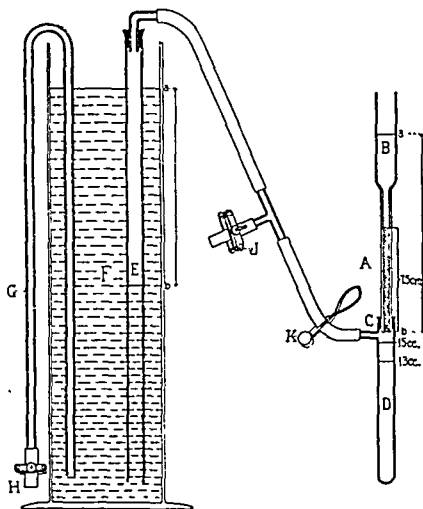


FIG. 1. Adsorption column

Down flow through the column in the analytical procedure should be so retarded that 5 ml. portions of the solution to be analyzed require 20 minutes or slightly longer to pass through. Various simple methods of controlling down flow were tried before the apparatus diagrammed in Fig. 1 was adopted. The lower tubing of the column, A, should have an internal diameter of 4 mm. or slightly less so that 1 ml. of fluid will form a column about 10 cm. long, and should be 12.5 cm. in length. A cotton plug to retain the resin is placed in the constricted tip of this tube. While the outlet of the tube is blocked with the finger, the column and reservoir are filled with 0.3 per cent sodium chloride solution. The resin is next introduced with the aid of a coarse pipette, being repeatedly tapped down to insure uniform packing, until it reaches a 7.5 cm. mark etched on the

column. The column is kept wet until used to prevent the formation of an air-lock. With a finger occluding the reservoir, *B*, of the column filled with 0.3 per cent sodium chloride, the column is attached by a rubber stopper, *C*, to a collecting tube, *D*, equipped with a side arm, which is temporarily closed by means of a pinch-cock on the attached rubber tubing. The other end of this rubber tubing is connected to a piece of glass tubing, *E*, roughly the same diameter as the collecting tube. This is inserted into a cylinder of water, *F*, in which the level of water can be adjusted by a syphon, *G*, and a screw-clamp, *H*.

With the apparatus thus prepared the saline above the neck of the reservoir, *B*, is removed by suction and replaced by 5 ml. of the solution to be analyzed. The cylinder is filled with water. By means of the T-tube, *J*, air is then blown into the system until the distance *ab* between the levels of water in the tube *E* and the cylinder equals the total height of fluid in the column *A* and the reservoir *B*. After equilibrium has been thus established, the pinch-cock, *K*, is opened. Now, by siphoning off the water from the cylinder *F* at the proper speed, the down flow of a battery of columns can be accurately regulated. When the initial 5 ml. portion has passed nearly through each column, clamp *K* is again closed, the reservoirs above the columns are filled with 0.3 per cent NaCl to marks calibrated to hold 5 ml., and enough water is added to the cylinder *F* to bring the level to a predetermined point required to establish equilibrium. This is repeated a second time to permit an accurately measured total volume of 13 ml. to pass through each column. These 13 ml. are then made up to 15 ml. of which 4 ml. are transferred to a colorimeter tube.

To the solutions in the colorimeter tubes, which must be chilled in advance in an ice bath, are added 1.2 ml. of fresh  $\alpha$ -naphthol-urea reagent. The contents of the tubes are mixed and the tubes are allowed to stand in the ice bath for 10 to 15 minutes. At the end of this time, 0.72 ml. of the sodium hypobromite solution is added in such a manner that prompt mixing is insured. The contents of the tubes are mixed with a rotary motion before these are replaced in the bath. Both the  $\alpha$ -naphthol reagent and the hypobromite solution must be kept chilled in the refrigerator.

The Evelyn photoelectric colorimeter with a 6 ml. aperture and a 540  $m\mu$  filter was used. Readings must be made at equal intervals after the addition of hypobromite. The color is fully developed at the end of 20 minutes, and at this time readings bear an almost linear relation to concentration up to 1.6 mg. per cent (*i.e.*, the reading equivalent to a concentration of 1.6 mg. per cent in the original solution). The blanks, however, become most uniform at approximately 60 minutes, at which time also the color produced by glycocyamine is particularly stable and intense. This is, therefore, the optimal time for reading the colors. The reference

curve for concentrations varying from 0.1 to 14 mg. per cent at the 60 minute interval is a smooth curve which becomes linear when plotted on paper with double logarithmic ordinates. Before reading the colorimeter tubes should be warmed gently with the hand for  $\frac{1}{2}$  minute and polished with a towel for an equal length of time.

Suitable standard solutions of glycocyamine in 0.3 per cent sodium chloride are analyzed simultaneously. The glycocyamine used in this study was synthesized by the method of Nencki and Sieber (12).

TABLE I

*Measurement of Glycocyamine in 0.3 Per Cent Sodium Chloride Solution, in Presence of Arginine*

The concentrations, expressed in mg. per cent, are those in the final mixtures before they were passed through the columns of resin. Standard solutions were introduced directly into the reservoir above the column, diluted to 5 cc. with sodium chloride solution, and mixed. A recent sample of analytical grade Amberlite, IR-100-Na, Batch 1943, was used throughout. In the experiments in which no resin was used the solutions were not passed through the columns. Instead 10 ml. of 0.3 per cent sodium chloride were added to the 5 ml. portions of standard solution, and aliquots of the mixture were taken for color development. Each figure represents the result of duplicate analyses which differed from one another by not more than 0.07 mg. per cent.

Added		Resin batch No.	Recovered glycocyamine
Glycocyamine	Arginine		
4.0	0	None	4.0
2.0	0	"	2.0
1.0	0	"	1.0
4.0	0	1943	3.97 (4.0)
4.0	25	1943	3.97 (4.0)
2.0	25	1943	1.99 (2.0)
2.0	0	1943	1.97 (1.98)
1.0	25	1943	1.00
0.0	25	1943	0.010

## EXPERIMENTAL

That added arginine can be completely removed from dilute sodium chloride solutions by this procedure, without appreciable loss of glycocyamine, is indicated by the data of Table I. An 0.3 per cent solution of sodium chloride was selected to simulate the quantity of salt to be expected in diluted dialysates of blood serum. Higher concentrations of salt tend to elute the arginine. Concentrations lower than 0.3 per cent do not affect recovery of glycocyamine, provided the 5 ml. portions are followed by 0.3 per cent sodium chloride. From urine diluted with 5 volumes of water

to which measured amounts of arginine and glycocyamine had been added, equally good separation was attained (Table II). Preliminary experiments with serum dialysates were undertaken, but could not be completed.

To test the reliability of the ion exchange resin, samples of Amberlite produced by the manufacturers over a period of several years were obtained. The lot numbers of these are given in Tables I and II. As these were available only in unrefined form, they were subjected to four sodium and regeneration cycles in a suitable column. It was found that they could be used interchangeably.

TABLE II

*Measurement of Glycocyamine Added, in Presence of Arginine, to Human Urine Diluted with 5 Volumes of Water*

The concentrations, expressed in mg. per cent, are those in the final mixtures before these were passed through the columns of resin. Standard solutions and controls not subjected to the action of resins were treated in the manner described in Table I. The numbers under the heading "Resin batch No." refer to the particular manufacturer's lot used in that analysis.

The specimen of urine used had a specific gravity of 1.021 and gave a 2+ reaction for albumin.

Added		Resin batch No.	Glycocyamine found	
Glycocyamine	Arginine		Total	Added
2.0	0	None	2.0	
1.0	0	"	1.0	
0.167	0	"	0.166	
0	0	1943	0.693	0
2.0	0	463	2.693	2.0
2.0	25	463	2.69	2.0
1.0	0	593	1.68	0.99
1.0	25	593	1.61	0.92
0.167	25	1943	0.86	0.17

The method of separation is in essence a form of colorless chromatography in which the glycocyamine is successively passed down the column of resin particles until it can be eluted in advance of the arginine. It is logical, therefore, that speed of down flow, salt concentration, and volume of eluant should require careful control.

The author's entry into military service prevented completion and amplification of this study.

## SUMMARY

A micromethod for the determination of glycocyamine and arginine in biological fluids is described. In contrast to previous methods, this pro-

cedure is accurately reproducible since it employs ion exchange resins with uniform properties.

A device to regulate down flow in small columns is described, which makes feasible chromatographic procedures involving the use of adsorbants of coarse texture.

The author is indebted to Dr. John P. Peters for his stimulation and encouragement and for editing this paper in the author's absence, and to Dr. Harold Cassidy, Dr. Alfred Wilhelmi, Dr. Werner Bergmann, and Dr. Abraham White for their advice. The persevering technical assistance of Dorothea Sims made possible the present partial completion of this work.

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# THE INHIBITION OF CARBOHYDRATE OXIDATIONS BY BORATE

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Boric acid has long been recognized as a reagent which combines with glycols, and in the case of sugars it has been employed to determine the steric configuration of hydroxyl groups (1). This is generally considered to be due to the capability of boron to combine preferentially with adjacent hydroxyl groups which bear a *cis* relation to one another.

The only attempt to employ this property of boron for the control or inhibition of reactions was reported by Myrbäck and Gyllensvärd (2) who inhibited the alkaline hypiodite oxidation of glucose and some of its derivatives with borate. They concluded that no oxidation could occur if adjacent hydroxyls on carbon atoms 1 and 2 combined with the boron atom, but that oxidation occurred when this possibility was excluded.

In exploring the room temperature reactions of some highly reactive sugars we have found that borate inhibition has a much greater applicability than a mere combination with adjacent hydroxyls would lead one to believe. Keto compounds, like 5-keto-D-gluconic acid, whose reactivity depends upon hydroxyls  $\alpha$  to a free keto group, are inhibited in many oxidative reactions. Dehydroascorbic acid, whose reactive center consists of two adjacent keto groups without any  $\alpha$  hydroxyls, is also inhibited. Likewise, the oxidation of 2,3-diketo-L-gulonic acid is inhibited by borate.

The results reported here in greater detail concern the inhibition by borate of 5-keto-D-gluconic acid, dehydro-L-ascorbic acid, and 2,3-diketo-L-gulonic acid upon oxidation in alkaline solutions with cupric ions, methylene blue, and molecular oxygen.

## EXPERIMENTAL

*Reduction of Copper*—1. Reagent. The reagent employed was the modified Benedict's reagent as previously described (3), and the determinations for copper reduction were carried out at 25° in the same manner. Additions of borate were made as sodium tetraborate decahydrate directly to the reagent. The quantities shown in Fig. 1 represent amounts added before the addition of sugar solution.

2. 5-Ketogluconic acid. This solution was made up to correspond to 50 mg. per ml. of calcium 5-ketogluconate according to previous directions (3).



3. Dehydroascorbic acid and 2,3-diketogulonic acid. 0.5000 gm. of ascorbic acid was oxidized with the required amount of iodine in aqueous potassium iodide solution and diluted to 100 ml. This solution upon standing for 12 to 18 days was employed as 2,3-diketogulonic acid, according to the conventional method of preparing this acid (4).

*Methylene Blue Reduction*—All reactions were carried out at 25° and solutions were brought to that temperature before use.

1. 5-Ketogluconic acid. Three solutions were made up: (a) methylene blue, 0.1000 gm. per 100 ml. of water, (b) sodium carbonate anhydrous, 5 gm. per 100 ml., and (c) 5-ketogluconic acid (see the reduction of copper). 3 ml. of solutions (b) and (c) were placed in a test-tube and 0.2 ml. of (a) added. The content was mixed by two complete inversions and an inch layer of benzene placed on top. The tube was then placed in the water bath and the time noted for disappearance of the blue color. Starting time was taken as the moment the tube was first inverted. Due to the concentration of methylene blue employed the end-point was not a complete disappearance of color; a slight shade of magenta remained.

Borate was added as sodium tetraborate decahydrate to the carbonate solution, and the quantities of Fig. 2 represent amounts placed in 3 ml. before the addition of sugar solution.

2. Dehydroascorbic acid. 0.2250 gm. of ascorbic acid was oxidized with the required amount of iodine in potassium iodide solution and diluted to 100 ml. with water. A sodium carbonate solution was made up, 5 gm. of the anhydrous salt per 100 ml., and methylene blue was used in the same concentration as with 5-ketogluconic acid.

3 ml. of the carbonate solution and 2 ml. of water were placed in a test-tube and 5 ml. of the dehydroascorbic acid solution added. Then 0.40 ml. of methylene blue solution was added and the content of the tube mixed by several inversions. An inch layer of benzene was placed on top. The tube was placed in the water bath and the time noted for disappearance of color, starting time being taken as the first inversion.

When borate was added, it was dissolved in the carbonate solution after the addition of water. The quantities of borate shown in Fig. 2 represent the amounts of sodium tetraborate decahydrate placed in 5 ml. before the addition of the sugar solution.

3. 2,3-Diketogulonic acid. The conditions were precisely the same as with dehydroascorbic acid, since the solution was prepared by allowing the dehydroascorbic acid solution to stand for 18 days.

*Oxidation with Oxygen*—Oxygen consumption was measured at room temperature in a standard macro Van Slyke amino nitrogen apparatus by placing a total of 20 ml. in the reaction chamber and shaking with pure oxygen at a rate of 235 complete oscillations per minute. Solutions employed were as follows.

1. 5-Ketogluconate. 0.5000 gm. of calcium 5-ketogluconate was decomposed with 1.500 gm. of oxalic acid dihydrate, and after filtration the solution was neutralized with 2.015 gm. of sodium bicarbonate before dilution to 100 ml. with water. Before introduction into the Van Slyke apparatus 10 ml. of this solution were mixed with 10 ml. of a solution of sodium carbonate made up, 15 gm. of anhydrous salt in 100 ml. of water.

For the inhibition by borate 200 mg. of sodium tetraborate decahydrate were added to 10 ml. of the carbonate solution before mixing.

2. Dehydroascorbic acid. To 20 ml. of a solution made up by oxidizing 0.5000 gm. of ascorbic acid with iodine (see the reduction of copper) were added 1.500 gm. of anhydrous sodium carbonate. Introduction into the apparatus was performed as rapidly as possible with a pipette. Inhibition by borate was effected by adding 0.5000 gm. of sodium tetraborate decahydrate with the carbonate.

3. 2,3-Diketogulonic acid. This solution was handled exactly as a dehydroascorbic acid solution. Its preparation consisted of allowing dehydroascorbic acid to stand for 18 days.

4. Ascorbic acid. 0.2500 gm. of ascorbic acid was dissolved in 10 ml. of water and introduced into the Van Slyke apparatus. 10 ml. of sodium carbonate solution (15 gm. of anhydrous salt in 100 ml. of water) were then introduced separately through the side arm of the Van Slyke apparatus. In the case of borate inhibition 0.2500 gm. of sodium tetraborate decahydrate was dissolved with the ascorbic acid.

### Results

In a former communication (3) the reaction between 5-ketogluconic acid and alkaline cupric ions was shown to have a velocity at 25° suitable for the quantitative determination of this compound. Ordinary salts such as sodium chloride, sodium nitrate, and others had no effect in changing the velocity.

Sodium tetraborate, however, when added to the ketogluconic acid cupric reaction, retarded the velocity markedly and if sufficient were added, the reaction was stopped completely. As an instance, a solution of 5-ketogluconic acid containing 50 mg. per ml. reduced the copper Benedict's reagent in 8 minutes and 20 seconds. The addition of 7 mg. of borate per ml. of reagent retarded the time to 24 minutes and 45 seconds; and the addition of 15 mg. per ml. stopped the reaction entirely—even for weeks. Fig. 1, Curve A, portrays the inhibition over various concentrations of borate until the reaction is completely stopped.

The character of Curve A is precisely that of a curve in which the concentration of 5-ketogluconic acid is progressively diminished. It would, therefore, seem that the borate is combining with the sugar and removing it from its rôle as a reducer. By calculating from a dilution curve such as

is employed in the determination of 5-ketogluconic acid (3), the quantity of acid inhibited by a given amount of borate can be obtained. The inhibition ratio of carbohydrate molecules to boron atoms is 8:1. This inhibition ratio remains the same throughout Curve A; but the significance of such a high figure can hardly be explained on the basis of simple combination.

Other carbohydrates that reduce Benedict's reagent at 25° are also inhibited by borate. In Fig. 1, Curve B, the results with dehydroascorbic acid and 2,3-diketogulonic acid are shown. The concentration-velocity curve for these two compounds is analogous to one in which the concentration of the compounds was plotted against velocity. At a concentration

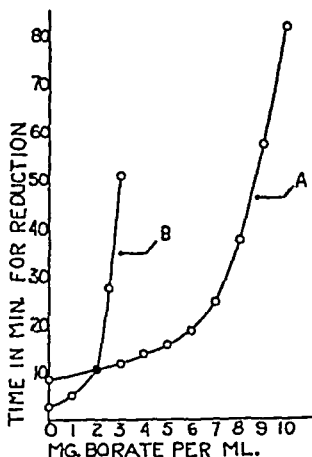


FIG. 1. The effect of borate on the alkaline copper reduction of (Curve A) 5-ketogluconic acid, 50 mg. per ml., and (Curve B) dehydroascorbic acid and 2,3-diketogulonic acid, 5 mg. per ml.

of borate which corresponds to a ratio of 6 sugar molecules to 1 boron atom, the reaction of these two intensely reactive compounds with cupric ions is completely inhibited.

Of all the structures we have investigated in relation to the reduction of cupric ions there are only two which are not inhibited by borate. Those are the enediol structure and the sulfhydryl group. Ascorbic acid cannot be inhibited from reducing cupric ions by a saturation of borate, whereas its oxidation products, dehydroascorbic acid and 2,3-diketogulonic acid, are completely inhibited. This effect is particularly noted in the titration of Benedict's reagent with a solution of ascorbic acid. In the absence of borate, titrations to the disappearance of blue color vary greatly among themselves (see Table I), depending upon the speed with which the ti-

tration is carried on. This is due to the reaction of dehydroascorbic acid with cupric ions, the rate of which is dependent on concentration. When borate is added to the reagent to inhibit this reaction, titrations are checked easily and can be used for the determination of ascorbic acid, just as the usual Benedict's method can be used for the determination of glucose. The method offers no advantages over existing methods due to the usual difficulty of the Benedict's end-point.

TABLE I

*Titration of 5 Ml. of Cold Benedict's Reagent with Ascorbic Acid (5.0 Mg. per Ml.)*

Without borate	With borate (0.3000 gm.)	Theory
ml.	ml.	ml.
3.80	6.55	6.40
4.36	6.50	
3.50	6.48	

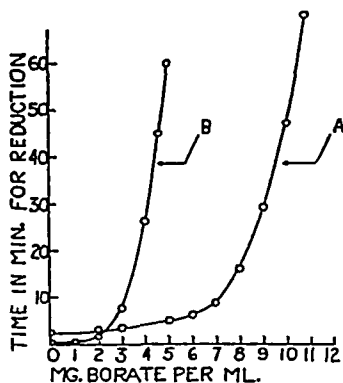


FIG. 2. The effect of borate on the alkaline methylene blue reduction of (Curve A) 5-ketogluconic acid, 50 mg. per ml., and (Curve B) dehydroascorbic acid and 2,3-diketogulononic acid, 5 mg. per ml.

From the inhibition of the copper reaction it could be supposed that borate combined either with the sugar or with the copper reagent. We, therefore, investigated other alkaline oxidation reactions which occur at lower temperatures (borate does not inhibit at high temperatures) for borate inhibition, employing the three sugars mentioned above. The picric acid, methylene blue, ferricyanide, and molecular oxygen reactions all proved to be inhibited by borate just as easily as the copper reaction. The picric acid reaction is so easily stopped that it was explored only qualitatively. The ferricyanide reaction will be reported elsewhere.

Fig. 2, Curve A, shows the inhibition caused by varying amounts of

borate added to an alkaline solution containing 5-ketogluconate and methylene blue. The curve follows the shape, as in the copper reaction, of one in which the concentration of the sugar is progressively diminished. Similarly, Curve B shows the inhibition of dehydroascorbic acid and of 2,3-diketogulonic acid. The curves of the latter two are so similar that they cannot be drawn separately on the same graph.

The oxidation by molecular oxygen is a reasonably rapid reaction at room temperature in alkaline solution with the compounds under consideration as illustrated in the third column of Table II. In the case of ascorbic acid the reaction is almost complete in  $\frac{1}{2}$  hour. It is generally supposed that the oxidation of sugars with oxygen involves an enolization prior to the reaction. If such is the case borate can prevent the formation,

TABLE II  
Oxygen Consumption in Alkaline Solution

Sugar	Concentration	Oxygen consumed by 20 ml.*	Borate added
	mg. per ml.	ml.	mg. per ml.
5-Ketogluconic acid .....	25	31.0 (5 hrs.)	0
" " .....	25	0 (5 " )	10
Dehydroascorbic " .....	5	10.9 (5 " )	0
" " .....	5	0.3 (5 " )	25
Diketogulonic acid .....	5	8.7 (1 hr.)	0
" " † .....	5	1.2 (3 hrs.)	25
Ascorbic acid .....	12.5	40.9 (2 " )	0
" " .....	12.5	24.4 (5 " )	12.5

\* At the times given oxygen consumption had practically ceased.

† Small amounts of ascorbic acid are no doubt present in this solution.

since it is capable of stopping the oxidation (see Table II) for long periods of time with all structures except the enediol. Enolization being a hydrogen migration, it would seem that borate is capable of combining with groups other than hydroxyl, such as keto groups. Especially must this be the case with dehydroascorbic acid in which the hydroxyl groups are some distance removed from the keto groups.

Ascorbic acid again presents an interesting case in the oxidation with molecular oxygen. Borate does not inhibit the reaction, but it does diminish the consumption.

One would expect the enediol structure of ascorbic acid to consume oxygen in the presence of borate, but the total consumed would be smaller because the oxidation products thereof are inhibited from reacting further with oxygen. The amount consumed in the presence of borate is greater than would be required for a stoichiometric reaction of the enediol group,

since the oxidation of ascorbic acid in strong alkaline solution is a complex reaction which cannot be written as a simple equation. This fact has been noted many times. The reaction mechanism is here complicated by the appearance of hydrogen peroxide as demonstrated by the work of Peterson and Walton (5).

#### SUMMARY

In alkaline solutions at ordinary temperatures the oxidations of 5-keto-D-gluconic acid, dehydro-L-ascorbic acid, and 2,3-diketo-L-gulonic acid are inhibited by borate ion strongly enough to stop the reactions completely. The inhibition is a general one and apparently involves a combination between the borate and the sugar.

Structures which are not inhibited by borate under the same conditions are the enediol, as in ascorbic acid, and sulfhydryl groups.

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# THE ENZYMATIC DEGRADATION OF THYMUS NUCLEOHISTONE

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In the course of studies on the antigenic composition of the more highly organized viruses and bacteria, it became apparent that the methods employed in the isolation of the desoxyribonucleoproteins of these forms were probably destructive of the native mode of organization of these substances. The most important antigenic substances, which in general do not appear to be the desoxyribonucleoproteins, frequently retain antigenicity after vigorous steps have been taken to free them from the organism. For example, several antigens, isolated from *Rickettsia prowazeki* after proteolytic digestion, emulated to a considerable extent the antigenic properties of the total organism, while nucleic acid and nucleoprotein fractions from these forms did not appear to possess specific serological reactivity (1). Since the possibility of degradation in the course of isolation was not excluded, a study was undertaken of the effect on a well known desoxyribonucleoprotein of various procedures for the fragmentation of an organism. The effects of one commonly used procedure, namely enzymatic degradation, are recorded in this paper. New analytical procedures for the estimation and characterization of nucleic acid, developed in this series of studies, have already been reported (2).

Thymus nucleohistone (TNH) was chosen as a model substrate for several reasons. The isolated nucleoprotein had been extensively studied with respect to various physicochemical properties (3-5), and several methods of preparation had been described (3, 6). Since one of these methods of preparation (6) is reported as being widely applicable in the isolation of desoxyribonucleoproteins, a careful comparison of the effects of the reagents used on the properties of TNH would be expected to yield information pertinent to our problem. In addition, it was considered that an examination of amino acids bound to nucleic acid after proteolytic digestion might yield some information concerning the structure of TNH and the mode of linkage between protein and nucleic acid.

Attention may be called to the inclusion of preparations of crystalline

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johnson Research Foundation, University of Pennsylvania.



ribonuclease in experiments comparing the proteolytic effects of different enzymes on TNH. In previous attempts to demonstrate ribose nucleic acid in cellular debris, incubation with preparations of ribonuclease,<sup>1</sup> followed by dialysis, resulted in the loss of nitrogenous material without the loss of phosphorus. These evidences of proteolytic activity for preparations of this enzyme have been observed by other workers (7). It was considered that a comparison of the effects of ribonuclease and other better known proteolytic enzymes on a single substrate would more exactly define the activity of the former.

#### EXPERIMENTAL

*Preparation of TNH, Histone, and Sodium Desoxyribonuclease (NaDN)*—The sodium salt of calf thymus nucleohistone was prepared as described by Carter and Hall (3). Solutions of freshly prepared undried TNH were employed in the proteolytic digestions. The Hammarsten procedure (8) was employed in the separation of nucleic acid and histone. Solutions of TNH were made molar with respect to sodium chloride and permitted to stand at 4° for a week. 2 volumes of 95 per cent ethyl alcohol were added and the sediment, after separation by centrifugation, was dissolved in M sodium chloride. The solution, clarified by centrifugation, was reprecipitated with alcohol. The precipitate of NaDN was washed with alcohol and ether, and dried over P<sub>2</sub>O<sub>5</sub> *in vacuo*. The first alcoholic supernatant solution was distilled *in vacuo* below 40° to remove the alcohol, and the histone solution was dialyzed against running tap water and distilled water. Preparations of TNH and histone were lyophilized and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub>.

Nitrogen was determined by the Kjeldahl method. Cupric sulfate was used as a catalyst during a 1 hour digestion period, and, after addition of 2 drops of 30 per cent hydrogen peroxide, the digestion was permitted to proceed for another hour. Shorter digestion periods yielded low results. Phosphorus was determined by the King procedure (9). Desoxyribose was determined by the diphenylamine procedure for purine desoxyribosides (10) and by the perchloric acid-tryptophane procedure for total desoxyribose (2). It was established by these procedures, in comparison with desoxyguanosine, that, in the preparations of TNH and NaDN, the molar ratio of purine desoxyribose to phosphorus was 0.5:1, and the ratio of total desoxyribose to phosphorus was 1:1, the maximum variations being 2 per cent from these theoretical ratios. Histone preparations contained a maximum of 1 per cent of nucleic acid, as contrasted to 42 to 46 per cent nucleic acid in TNH. Preparations of NaDN had a molar ratio of

<sup>1</sup> The author is indebted to Dr. M. Kunitz of the Rockefeller Institute for the crystalline enzyme preparations employed.

nitrogen to phosphorus having a maximum deviation of 2 per cent from the theoretical ratio of 3.75.

*Proteolytic Digestion of TNH*—The viscosity changes as a result of proteolysis and the composition of the digested nucleoproteins, after removal of dialyzable amino acids and protein fragments, have been determined. In a typical experiment, 75 cc. of TNH at a concentration of histone<sup>2</sup> of 0.450 mg. of N per cc. in 0.1 M. borate buffer at pH 7.80 containing 0.01 per cent ethyl mercurithiosalicylate were added to each of five flasks. To these solutions were added 7 cc. either of water or of 0.1 per cent solutions of Fairchild trypsin or of crystalline preparations of ribonuclease, chymotrypsin, and trypsin. The mixtures were incubated at 37° for 48 hours and dialyzed against running tap water and distilled water. Portions of this solution were lyophilized and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub>.

*Analyses*—The white fibrous solids were analyzed for nitrogen and phosphorus. Tyrosine was estimated in alkaline hydrolysates by the Bernhart method (11). Arginine was determined by the procedure of Brand and Kassell (12). In analyses for the arginine content of these materials, there was no inhibition of color development with increasing concentrations of hydrolysate. In one set of digested products, residual phenylalanine was also estimated (13).

The specific viscosity of solutions of these products in M sodium chloride was determined in an Ostwald viscosimeter at 28° at concentrations of 0.02 mg. of phosphorus per cc. These data are presented in Table I for the preparations described, which are designated TNH-I. These analyses and that of another set of products are contrasted in Table II in terms of the molar relationship of various residual moieties to phosphorus. The phosphorus-containing component, desoxyribose nucleic acid, was not lost in these experiments.

*Viscosimetry*—5 cc. aliquots of TNH-I and enzymes, prepared as described previously, were permitted to digest at 37.0° ± 0.1° in an Ostwald viscosimeter for 48 hours and the specific viscosities were determined at suitable intervals. The marked viscosity changes are presented in Fig. 1.

Dilutions of solutions of the undried, digested, and dialyzed products were examined in this apparatus. In solutions of less than 0.07 mg. of P per cc., specific viscosity apparently varied linearly with concentration.

<sup>2</sup> In the majority of experiments described in this section, TNH solutions were adjusted to an appropriate histone N concentration and the rates of digestion of TNH and histone solutions of the same histone content were compared by a variety of methods. These data are not considered sufficiently complete and will not be discussed further. It may be noted that ribonuclease degraded free histone as well as TNH.

## DEGRADATION OF NUCLEOHISTONE

In contrast to the identity of specific viscosities in M sodium chloride, the specific viscosity of these substances in water could be correlated with their decreasing N:P ratios or amounts of residual protein. These data are presented in Table III.

TABLE I  
Properties of Proteolytically Digested Thymus Nucleohistone-I

Analysis	TNH-B*	TNH-R	TNH-CT	TNH-T	TNH-FT
Nitrogen, %	16.04	15.60	15.08	14.68	14.38
Phosphorus, %	4.20	5.36	6.25	7.00	6.85
Tyrosine, %	1.90	1.59	0.46	0.88	0.65
Arginine, %	7.21	6.51	5.02	2.94	3.18
Spontaneous birefringence†	—	±	3+	3+	3+
Specific viscosity‡	0.054	0.052	0.054	0.053	0.056

\* B, R, CT, T, and FT refer to treatment with water, ribonuclease, chymotrypsin, trypsin, and Fairchild trypsin respectively.

† Examined as a solid in the laboratory of Dr. I. Fankuel en of the Brooklyn Polytechnic Institute.

‡ Estimated at 0.02 mg. of P per cc. in M sodium chloride at 23°.

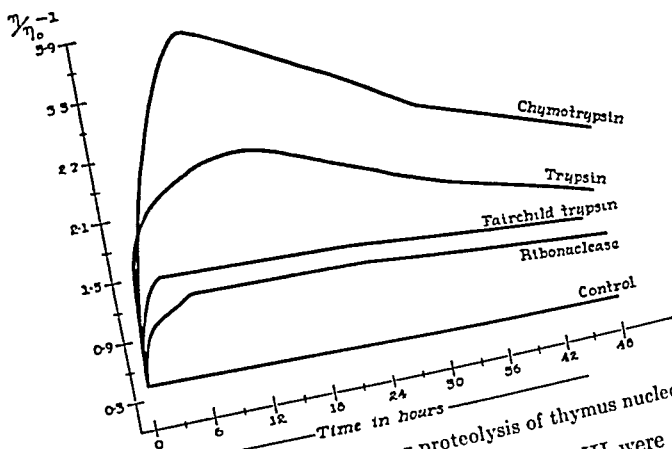


FIG. 1. Specific viscosity changes during proteolysis of thymus nucleohistone-I. When solutions of freshly prepared undialyzed TNH were made molar with respect to sodium chloride, their viscosities increased markedly. The dependence of viscosity upon concentration was no longer linear, but was now indicative of considerable particle interaction. Furthermore, al-

though renewed dialysis of a dialyzed digested product against distilled water did not result in the further loss of amino acids, dialysis in *M* sodium chloride, against *M* sodium chloride, now resulted in the free diffusion of arginine, but not of nucleic acid, through the membrane.

TABLE II

*Residual Protein Constituents in Proteolytically Digested Thymus Nucleohistone*

Substances*	Preparation No.	Molar ratios			
		N:P	Tyrosine to P	Arginine to P	Phenylalanine to P
TNH-B*	1	8.44	0.075	0.307	
	2	8.04	0.074	0.217	0.095
TNH-R	1	6.43	0.051	0.216	
	2	5.53	0.035	0.062	0.090
TNH-CT	1	5.32	0.013	0.143	
	2	4.91	0.014	0.043	0.047
TNH-T	1	4.64	0.022	0.075	
	2	4.84	0.028	0.023	0.006
TNH-FT	1	4.62	0.016	0.033	
	2	4.11	0.000	0.016	0.000

\* The letters, B, R, etc., possess the same significance as in Table I. Preparations 1 and 2 are derived from TNH-I and TNH-II. The ratios of tyrosine and phenylalanine to phosphorus of TNH-B2 were identical with those of TNH-II.

TABLE III

*Viscosity of Digestion Products of Thymus Nucleohistone-I*

Substance	Molar ratio, protein N:P	Specific viscosity in water per 0.05 mg. P	Per cent loss of specific viscosity by rabbit sera in 1 hr.
TNH-B	4.69	0.194	30
TNH-R	2.68	0.500	56
TNH-CT	1.57	0.550	52
TNH-T	0.79	0.559	76
TNH-FT	0.77	0.690	53

*Action of Thymonucleodepolymerase in Rabbit Sera (14)*—1 cc. aliquots of rabbit sera, diluted with an equal volume of saline, were added to 4 cc. aliquots of the digested TNH-I products in the absence of additional electrolyte or in *M* sodium chloride. The change in viscosity at 37° was determined for a 6 hour period. Sera inactivated at 65° did not appreciably affect the viscosity of the TNH products at 37°, eliminating the possibility that the rapid decreases observed were due to proteolytic degradation of the diluted sera. Decreases were not observed in *M* sodium

chloride, whether the substrates were TNH products or NaDN. The percentage loss of specific viscosity after 1 hour as contrasted to the total 6 hour decrease is given in Table III.

#### DISCUSSION

From the data presented above, several conclusions may be drawn. It may be expected that in an antigenic analysis of cellular systems, procedures involving proteolytic digestion or autolysis probably result in the degradation of the desoxyribonucleoproteins. The rôle of these substances in an immune response is most obscure. The problem of preparing these nucleoproteins in an indisputably native state for immunological studies has not yet been solved.

*Proteolysis of Chromatin*—Nucleoproteins have been demonstrated to be a most important component of chromatin, and the degradation by proteases of those portions of chromosomes apparently bound to nucleic acid has been known for some time. This treatment, if carried to completion in the absence of a nucleic acid precipitant such as lanthanum ion, results in the disintegration of the chromosome (15). In these *in vitro* tests, proteolytic degradation of TNH resulted in increased interaction of freed portions of nucleic acid; *i.e.*, disaggregation within molecules of TNH resulted in increased aggregation of the partially degraded molecules on an intermolecular level. Thus protein movement from a portion of a chromosome might result in a localized increased interaction of residual nucleic acid, preserving the apparently quiescent gross form of the chromosome whose metabolism and internal transformations may be intensely active at the moment considered. The ease of degradation of TNH by various proteolytic enzymes is in marked contrast to the inability of these same enzymes to degrade the few well defined ribonucleoproteins, the plant viruses (16-18).

*Ribonuclease As Proteolytic Enzyme*—The demonstration that the preparations of ribonuclease have a marked proteolytic effect on TNH and histone raises some interesting points. This effect is manifested by the removal of considerable amounts of arginine and tyrosine and only small amounts, if any, of phenylalanine from the nucleoprotein, as well as by marked viscosity changes characteristic of proteolysis by other enzymes. A comparison of the proteolysis effected by ribonuclease with those of the other enzymes appears to indicate a considerable degree of dissimilarity in the mechanism of their action. It is impossible to say at present whether the proteolytic activity of ribonuclease preparations is an intrinsic property of the ribonuclease molecule or is due to the presence of a contaminant. Similar preparations have been used by numerous workers to demonstrate the presence of ribonucleic acid in nucleoli, in the hulls of Gram positive

bacteria, in cytoplasmic bodies, etc. It is necessary to ask whether the loss of nucleotide properties, considered by these workers to be of ribonucleic acid origin, may not be a non-specific loss of nucleotides, such as adenylic acid, due to the proteolytic degradation of the protein to which they were bound.

*Specificity of Proteolysis*—An examination of Table II, presenting the molar ratios of amino acid to phosphorus in the two sets of TNH and the degradation products, reveals some interesting correlations. In the control nucleohistones, TNH-B1 and TNH-B2, the difference in arginine to P ratios ( $0.307 - 0.217 = 0.090$ ) is equivalent when converted to N:P by multiplying by 4, to a difference of 0.36 in the N:P ratios. The actual difference found in the latter is 0.40. Thus within two preparations of TNH, the variation in arginine content accounts for at least 90 per cent of the total variation in the nitrogen content. In contrast to the results on arginine, the tyrosine to P ratios are practically identical.

In the derived preparations of TNH-CT, the difference in the arginine to P ratio accounts even more precisely for the difference in N:P, while the tyrosine to P ratios are again identical. Furthermore, the differences in arginine to P and N:P, 0.100 and 0.41 respectively, observed for these degraded products are essentially those of the starting materials. In this system, therefore, the variable arginine of TNH represented by these differences not only is not removed by degradation but does not affect the course of degradation, at least with respect to the amount of tyrosine removed and presumably of the other amino acids as well.

In contrast to the above, when the degradations are accomplished with ribonuclease, trypsin, and Fairchild trypsin, it may be seen that the initial arginine content affects the quantity and quality of degradation with respect to arginine, tyrosine, and total nitrogen. This phenomenon is most marked with trypsin. Thus the difference in arginine content in the starting materials would appear to affect the course of hydrolysis for some enzymes but not for others. Since it is known that trypsin is reactive with peptide linkages involving the carbonyl group of a basic amino acid, it might be expected that this enzyme would be more sensitive to differences in arginine content than is chymotrypsin. The latter is reactive with peptide linkages involving the carbonyl groups of aromatic amino acids (19), in addition to the other specificities which it possesses (20).

*Depolymerization of Free and Bound Nucleic Acid*—When employed in purified systems containing ribonucleic acid, ribonuclease will apparently depolymerize free ribonucleic acid but not ribonucleoproteins (18). In the case of the crude desoxyribonucleodepolymerase of rabbit sera, the depolymerization of nucleohistone, nucleic acid, and of all the intermediate proteolytically degraded products, occurs with considerable facility. The

inability of this enzyme to act in  $M$  sodium chloride is perhaps another indication of the dissociating effect of this concentration of electrolyte on nucleic acid-protein linkages. The combination of this substrate and enzyme may be inhibited by solutions of high ionic strength. On the other hand, a small amount of dissociation at low ionic strength may make the nucleic acid from TNH and products available for depolymerization by this enzyme.

*Dissociability of Nucleohistone in Concentrated Electrolyte*—A considerable portion of the data presented points to the dissociating effect of  $M$  sodium chloride on TNH. This raises questions concerning the use of this solvent as a general method (6, 21) for the preparation of desoxyribonucleoproteins in a mode of organization closely approximating the native relationship of nucleic acid and protein. These data may be summarized as follows.

Polymerized NaDN may be prepared by the solution of TNH in  $M$  sodium chloride, followed by precipitation of separated NaDN with alcohol. This separation does not occur to the same extent in the absence of salt.

The high viscosity of solutions of NaDN is approached by products resulting from proteolytic degradation of TNH. The specific viscosity in water of the degraded products at the same  $P$  concentration is approximately in the order of increasing nucleic acid concentration within the substance studied.

The viscosity of solutions of undialyzed TNH is markedly increased to a value approaching that of polymerized NaDN, following adjustment to molarity with respect to sodium chloride.

The viscosities of solutions of TNH and its degraded products in  $M$  sodium chloride are essentially identical at the same nucleic acid concentration.

Degraded nucleoproteins, completely dialyzed in water, yield dialyzable amino acids in  $M$  sodium chloride. The dialyzability of protamine from nucleoprotamines in the latter solvent has been demonstrated by Mirsky and Pollister (6).

This summation may be interpreted as demonstrating that removal of protein from TNH by digestion produces effects which may be extrapolated to the effects observed in  $M$  sodium chloride. That is, the polymerization phenomena observed in both systems are completely explicable in terms of the liberation of nucleic acid moieties available for interaction and aggregation. Finally, it appears that the partial liberation of nucleic acid effected by proteolysis may be carried to completion at any stage by  $M$  sodium chloride.

It would appear to be established that the dissociability of desoxyribonucleoproteins in  $M$  sodium chloride is considerably greater than in solutions

of low electrolyte concentration. If the general method of preparation proposed (6, 21) involves extraction of tissue with the former and precipitation with the latter, it is not unlikely that a considerable reshuffling of the two original components, nucleic acid and protein, occurs with each reprecipitation. Furthermore, the combination of protein not originally present in the complex becomes possible. If one is concerned with studies of chromatin and its various activities, it is not considered that materials prepared by this method would behave in a manner similar to the original chromatin, since the mode of organization has probably been altered.

#### SUMMARY

Preparations of thymus nucleohistone have been digested with various proteolytic enzymes. The changes in amino acid composition and viscosity as a result of proteolytic degradation have been presented. It has been demonstrated that crystalline preparations of ribonuclease have proteolytic activity. The degradation of desoxyribonucleoproteins as well as desoxyribonucleic acid by the thymonucleodepolymerase in rabbit sera has been described. It has been shown that the nucleic acid-protein linkage in these substances is dissociated to a greater extent in  $M$  sodium chloride than in water. This phenomenon has been correlated with viscosity effects attendant on the freeing of nucleic acid by the removal of protein, permitting increased nucleic acid interaction. The implications of these findings for the preparation of desoxyribonucleoproteins have been discussed.

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# CHEMICAL DETERMINATION AND URINARY EXCRETION OF THE METABOLITE N<sup>1</sup>-METHYLNICOTINAMIDE

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Independent studies in two laboratories have led to the development of chemical tests for the early diagnosis of pellagra. Melnick and associates (1-3) reported the excretion in urine of a compound which fails to react like nicotinic acid or amide with cyanogen bromide and aniline (Koenig reaction) unless previously subjected to alkaline hydrolysis. The physical and chemical similarity of the metabolite to trigonelline, the anhydride of N<sup>1</sup>-methylnicotinic acid, suggested that the latter may be a normal urinary constituent derived in part from dietary sources. With increasing dosage of either nicotinic acid or nicotinamide, there was a parallel increase in the urinary excretion of the metabolite. Pellagrins were observed to excrete only small quantities of this substance, a finding which led to the development of a standardized urinary excretion test for the early detection of nicotinic acid deficiency. Using modifications in the basic analytical techniques, a number of investigators (4-7) reported confirmatory data and similar conclusions.

Najjar and associates (8-11), on the other hand, approached this problem through an unrelated analytical procedure. They reported that normal subjects excreted appreciable quantities of a compound, readily converted by alkalization and subsequent extraction with butanol to a highly fluorescent substance. Only small amounts of this compound were found in the urine of nicotinic acid-deficient subjects. The variability in response following nicotinic acid or amide dosage paralleled the basal urinary excretion. It was subsequently reported by Huff and Perlzweig (12) that the urinary metabolite was N<sup>1</sup>-methylnicotinamide. In addition, the latter workers demonstrated that the compound responsible for the positive Koenig reaction after alkaline cleavage was N<sup>1</sup>-methylnicotinamide rather than the anhydride of N<sup>1</sup>-methylnicotinic acid (trigonelline). Thus, two entirely different methods for the detection of nicotinic acid deficiency, the colorimetric (3, 6) and the fluorometric (9, 11), are actually dependent upon the measurement of the same compound.

Because of its greater precision the fluorometric procedure lends itself more readily to quantitative standardization. In our studies with normal subjects on the availability of the water-soluble vitamins (13), excellent correlation was found between the urinary excretion of the vitamins (or

derivatives) and intake. However, the attainment of such results in studies with nicotinamide was only possible when Najjar's method (8, 11) was improved to permit quantitative recoveries of added N<sup>1</sup>-methyl-nicotinamide.

In the present paper are presented a modification of the fluorometric procedure and data on the urinary excretion of N<sup>1</sup>-methylnicotinamide by normal subjects before and after nicotinamide dosage. All values for the urinary excretion of the metabolite were obtained with N<sup>1</sup>-methyl-nicotinamide chloride as the reference standard. However, the results are more conveniently expressed in terms of nicotinamide on an equimolar basis, especially since the exact structure of the metabolite is in dispute. One group of investigators believes it to be the chloride (12), while another considers it to be the carbinol (14). Only in the experiments dealing with a critical evaluation of the analytical method involving recovery tests, etc., are the results expressed in terms of the reference standard, N<sup>1</sup>-methylnicotinamide chloride.

#### EXPERIMENTAL

Synthetic N<sup>1</sup>-methylnicotinamide chloride was prepared as described by Huff and Perlzweig (12). The white, crystalline compound melted at 230° and its picrate at 186°, both uncorrected. When tested by the alkalization procedure described in this report, the fluorescence of 25  $\gamma$  produced a deflection of approximately 75 galvanometer units on the Pfaltz and Bauer fluorophotometer.<sup>1</sup>

In Fig. 1 are presented the ultraviolet absorption curves of the crystalline N<sup>1</sup>-methylnicotinamide chloride and U. S. P. nicotinamide in aqueous solution at pH 9.7. The data were obtained with a calibrated Beckman spectrophotometer with the hydrogen discharge tube and calibrated quartz accessories.<sup>2</sup> Extinction ratios,<sup>3</sup> *i.e.* the ratios of the extinction coefficients at each wave-length to that at the absorption maximum, are plotted from 240 to 300  $m\mu$ .

The absorption curves differ considerably from those reported by Huff and Perlzweig (12); they do agree, however, in the position of the absorption maxima for the two compounds. A sample of N<sup>1</sup>-methylnicotinamide chloride, now used for reference purposes by Dr. V. A. Najjar of the Johns

<sup>1</sup> The fluorometer and filters are the same as those used in determination of vitamin B<sub>1</sub> by the thiochrome procedure (15). They may be obtained from Pfaltz and Bauer, Inc., Empire State Building, New York.

<sup>2</sup> Manufactured by the National Technical Laboratories, South Pasadena, California.

<sup>3</sup> Plotting ratios rather than extinction coefficients yields absorption curves which are independent of the concentrations of test materials.

Hopkins Hospital, gave exactly the same absorption data as the sample prepared in our laboratories.

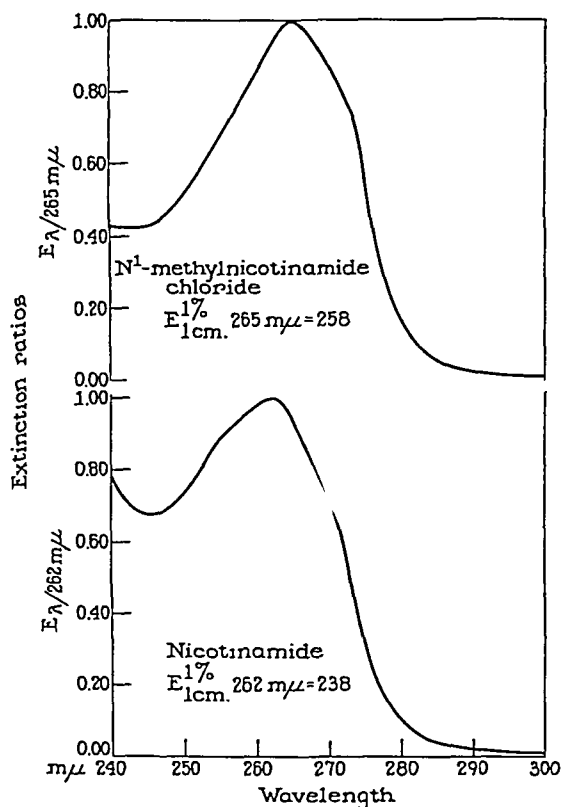


Fig. 1. Ultraviolet absorption curves of N¹-methylnicotinamide chloride and nicotinamide in aqueous solution buffered at pH 9.7 with carbonate-bicarbonate buffer.

### Method

The method employed for the determination of N¹-methylnicotinamide in urine involves adsorption of a small aliquot at pH 4.5 on a column of synthetic zeolite, elution with potassium chloride, alkalization, and extraction of the resulting fluorescent compound with normal butanol. The fluorescence is measured and compared with that obtained with a standard solution of N¹-methylnicotinamide carried through the entire procedure.

A blank correction is made for the possible presence of butanol-soluble fluorescent compounds in the eluate prior to alkalization.

*Apparatus—*

Fluorometer. In these studies a Pfaltz and Bauer model A fluorophotometer was used with a Corning red ultra No. 5840 filter for the

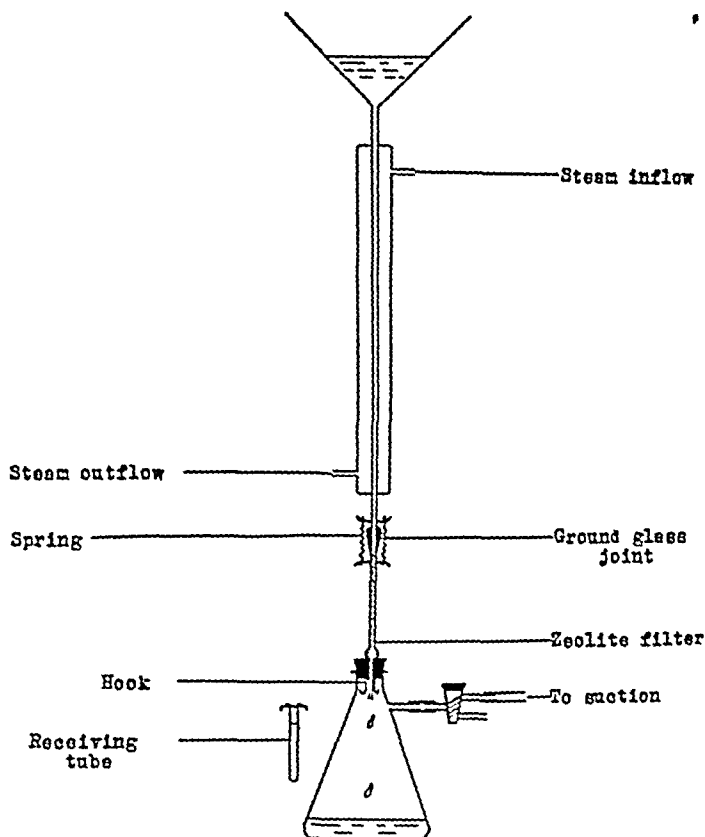


FIG. 2. Apparatus for the adsorption and elution of N<sup>1</sup>-methylnicotinamide in urine.

irradiating light beam, and two filters for the fluorescent light. The latter were a straw-yellow noviol shade A, No. 038, and a light shade blue-green, No. 428. Any similar instrument may be employed.<sup>1</sup>

Adsorption column. The apparatus used for the adsorption and elution of the N<sup>1</sup>-methylnicotinamide chloride in urine is shown in Fig. 2. Precautions have been taken in the construction of the apparatus to prevent

contamination of the samples with fluorescent substances present in rubber. A glass funnel is sealed to the top of a condenser. This is connected by means of a ground glass joint to a zeolite filter. The latter is a tube having an internal diameter of 8 mm. for a length of 4.5 inches beyond the joint, followed by a constricted portion about 4 mm. in diameter. A plug of glass wool is placed at the top of the constriction, and the tube is filled with the treated zeolite to a height of 4 inches; this requires about 3 gm. of the adsorbent. The zeolite filter is fastened to the bottom of the condenser by two 2 inch steel springs, joined to glass hooks. At the bottom of the apparatus is a 2 liter suction flask which acts as a reservoir. This is equipped with a 2-way stop-cock for connection to suction or to the atmosphere. Also shown is a receiving tube, calibrated at the 15 ml. mark. This is suspended from glass hooks in the stopper of the flask to collect the eluate. The condenser, which acts at times as a steam jacket, is connected to a laboratory steam line, or to a 1 liter flask of boiling water equipped with a 2-way stop-cock.

Alkalinization vessels.<sup>4</sup> 30 ml. separatory funnels or glass-stoppered centrifuge tubes. It is preferable not to use a stop-cock lubricant; however, if used, it should be tested for absence of fluorescence in butanol before and after alkalinization.

#### *Reagents—*

Standard solution. 50.0 mg. of N<sup>1</sup>-methylnicotinamide chloride<sup>5</sup> are dissolved in about 500 ml. of distilled water containing 0.5 ml. of concentrated hydrochloric acid. Distilled water is added to bring the volume to 1000 ml. The solution, preserved in the refrigerator in a glass-stoppered amber bottle, is stable for at least 1 month. The standard contains 50  $\gamma$  of N<sup>1</sup>-methylnicotinamide chloride per ml., equivalent to 35.4  $\gamma$  of nicotinamide on a molar basis.

Working fluorescent standard. A fluorescent glass block<sup>6</sup> has been found more convenient and reproducible as a secondary standard than the usual quinine sulfate solution (17). When used with the instrument and filters described in the present report, the block has a fluorescent intensity equivalent to that of 0.3  $\gamma$  of quinine sulfate per ml. of 0.1 N sulfuric acid.

<sup>4</sup> These are the vessels in which the oxidation of thiamine to thiochrome takes place in the fluorometric method for estimating thiamine (15). They may be obtained from Pfaltz and Bauer, Inc., New York.

<sup>5</sup> The compound may be prepared by the method of Huff and Perlzweig (12) or Karrer et al. (16) or may be purchased from W. A. Taylor and Company, 7300 York Road, Baltimore, Maryland.

<sup>6</sup> Obtained from the Bausch and Lomb Optical Company, Rochester, New York. The block is used merely to set the fluorometer each time a reading is taken. A primary standard of N<sup>1</sup>-methylnicotinamide is carried through the entire procedure to calibrate the instrument each day.

Acetate buffer at pH 4.5. 15.0 gm. of sodium acetate are dissolved in 2.5 liters of distilled water and 2.0 ml. of concentrated sulfuric acid are added.

Zeolite.<sup>7</sup> Approximately 50 mesh. This is first prepared in bulk by stirring with four 10 volume portions of 3 per cent acetic acid for 10 minutes each. Between the second and third acid wash, a 15 minute treatment with 5 volumes of neutral, 25 per cent potassium chloride is introduced. The zeolite is washed consecutively with water, alcohol, and ether, and then dried in air and stored in a sealed bottle.

Eluting solution. 250 gm. of potassium chloride are dissolved in sufficient distilled water to make 1000 ml.

Alkalinizing solution. 75 gm. of sodium hydroxide are dissolved in sufficient distilled water to make 500 ml.

*n*-Butanol, c.p. The solvent should be checked for fluorescence. After use, it may be recovered by distillation; charcoal treatment is not suitable. To prevent contamination with fluorescent impurities, contact with rubber should be carefully avoided.

Anhydrous sodium sulfate, c.p. The commercially available anhydrous salt should be dried overnight at 110° and stored in a tightly closed container.

### Procedure

A 24 hour urine sample is collected in a bottle containing 20 ml. of 3.5 N sulfuric acid. The total volume is measured. A 6 minute aliquot<sup>8</sup> is diluted with the acetate buffer to 50 ml. The solution is passed through the adsorption column at room temperature. A filtration rate of approximately 2 drops per second is easily maintained by the application of mild suction, controlled by means of the 2-way stop-cock. Steam is then passed through the outside jacket, and 30 ml. of water are poured on the column. This is allowed to heat for half a minute and is then drawn completely through with full suction in order to heat the zeolite adsorbate. The N<sup>1</sup>-methylnicotinamide is eluted immediately by passing 15 ml. of the potassium chloride solution down the wall of the hot condenser. The eluate is collected in the receiving tube at the rate of approximately 1 drop per 2 seconds. The last few drops are drawn through by suction.

The zeolite column is then washed with 300 ml. of distilled water, while

<sup>7</sup> Decalso, obtained from The Permutit Company, New York. Zeolite of the proper mesh should be used, since adsorption and elution of the N<sup>1</sup>-methylnicotinamide are functions of the area of the particles exposed to the solutions.

<sup>8</sup> For normal subjects on a basal diet containing approximately 20 mg. of nicotinamide, a 6 minute sample is taken. After dosage with 100 mg. of nicotinamide, a 2 minute aliquot is sufficient. In no case should more than a 6 minute sample be assayed.

steam passes through the jacket. The column is finally cooled to room temperature by running the last 75 ml. of wash water through with the steam turned off. The apparatus is then ready for the next sample. A standard solution containing 50  $\gamma$  of N<sup>1</sup>-methylnicotinamide chloride in 50 ml. of acetate buffer at pH 4.5 is passed through the column in the same manner as the diluted urine. A single zeolite column is used for all urine samples to be tested on a given day; these may be as many as fifteen samples.

The eluate is mixed and tested as follows: 5.0 ml. are pipetted into each of the two 30 ml. separatory funnels or the glass-stoppered centrifuge tubes. To one, the blank, 1 ml. of water is added, followed by 16.5 ml. of *n*-butanol. To the other vessel, the same volume of *n*-butanol is added, then 1 ml. of the alkalinizing solution. The vessels are stoppered immediately and shaken vigorously for 3 minutes. The water and butanol phases are separated by low speed centrifugation for half a minute. The aqueous layer is drawn off and discarded and the butanol phase is shaken with about 1 gm. of anhydrous sodium sulfate to remove the last traces of water. After 15 minutes, during which time the fluorescence reaches a maximum, the butanol extracts are read in a fluorophotometer. When the Pfaltz and Bauer model A fluorophotometer is used, the diaphragm is adjusted so that the glass fluorescent block produces a galvanometer deflection of 60 units. The block is used only for the setting of the instrument before each reading. A test with a standard solution of N<sup>1</sup>-methylnicotinamide chloride carried through the entire procedure must be conducted with each series of samples analyzed.

*Calculation*—The 24 hour urinary excretion of N<sup>1</sup>-methylnicotinamide chloride expressed in terms of nicotinamide is calculated as follows:

$$50 \gamma \times \frac{Gu}{Gs} \times \frac{60 \text{ minutes}}{M \text{ minutes}} \times 24 \text{ hours} \times 0.707 = \text{micrograms per day}$$

*Gu* and *Gs* are the galvanometer deflections of the unknown and the standard, both corrected for their respective blanks. *M* is the size of the urinary aliquot tested, measured in minutes. 0.707 is the factor for conversion of N<sup>1</sup>-methylnicotinamide chloride to nicotinamide.

*Evaluation of Method*—Varying aliquots of a composite urine sample with and without added N<sup>1</sup>-methylnicotinamide chloride were tested by the method described above. The results are presented in Table I. Maximal initial values and complete recoveries were obtained with 6 minute aliquots or less. However, both the initial values and the recoveries decreased progressively when aliquots of more than 6 minutes were tested. In some cases recoveries as low as 30 per cent were obtained when 60 minute urine aliquots were passed through the zeolite column. In the case of normal



subjects on an adequate basal diet containing approximately 20 mg. of nicotinamide per day, the galvanometer deflection after alkalization of a 6 minute eluate is approximately 20 units, a value sufficiently large for precise measurement. The reproducibility of the values obtained in replicate assays is within  $\pm 5$  per cent of the average figure.

The complete recoveries of the added N<sup>1</sup>-methylnicotinamide chloride noted above are obtained when comparisons are made with pure solution standards that have passed through the zeolite column. However, the fluorescence obtained by alkalization of a zeolite eluate is approximately 85 per cent of that obtained with a 25 per cent potassium chloride solution containing the same initial concentration of N<sup>1</sup>-methylnicotinamide chloride

TABLE I

*Effect of Size of Urine Aliquot on Initial Value and Recovery of Added N<sup>1</sup>-Methylnicotinamide Chloride*

Tests were conducted on a composite of five 24 hour urine samples obtained from five normal, male adults subsisting on a ration furnishing 23 mg. of nicotinic acid (predominantly as the amide) per day. Aliquots of the urine were buffered at pH 4.5 and passed through the zeolite column.

Aliquot	Basal excretion		Recovery of added N <sup>1</sup> -methylnicotinamide chloride	
	Found	Per cent of maximal value	Total found (8.0 mg. per 24 hrs. added)	Recovery of added compound
min.	mg. per 24 hrs.		mg. per 24 hrs.	per cent
60	5.8	67	12.0	77
40	6.6	77	13.0	80
25	7.5	87	14.1	83
12	7.8	91	15.4	95
6	8.6	100	16.9	104
3	8.6	100	16.6	100

before passing through the column. In an attempt to explain this apparent loss, the fate of the compound during the zeolite adsorption-elution step was investigated. 100  $\gamma$  in a volume of 25 ml. of acetate buffer were passed through the column. The filtrate and 25 ml. washing were collected independently. 25 ml. of potassium chloride were employed for the elution and the eluate collected in successive 5 ml. fractions. The results are given in Table II. Only negligible amounts of N<sup>1</sup>-methylnicotinamide chloride were present in the filtrate and the washings of the zeolite column. 84  $\gamma$  were found in the first 15 ml. of eluate, with 3  $\gamma$  in the next 5 ml., and a negligible amount in the next portion. Recoveries of 81 to 86 per cent are obtained routinely when the recommended 15 ml. are used for the elution, regardless of whether the column is being employed for the first or tenth sample and whether the tests are conducted with the compound

in pure solution or added to 6 minute urine aliquots. Though these experiments do not indicate the fate of the missing 15 per cent, the results stress the importance of carrying the standard through the zeolite step to insure proper evaluation of the results.

*Urinary Excretion of N<sup>1</sup>-Methylnicotinamide by Normal Subjects*—Data on the urinary excretion of N<sup>1</sup>-methylnicotinamide by eight normal male subjects are presented in Table III. These were laboratory workers with good dietary histories. On test days an adequate diet containing (19) by

TABLE II

*Fractionation of N<sup>1</sup>-Methylnicotinamide Chloride in Adsorption-Elution Step*

100  $\gamma$  of N<sup>1</sup>-methylnicotinamide in 25 ml of acetate buffer at pH 4.5 were passed through the column. The adsorbate was washed with 25 ml of hot water

Fraction	N <sup>1</sup> -Methylnicotinamide
	per cent*
Filtrate†	0.6
Washing†	0.3
Eluate, 1st 5 ml	51
2nd 5 "	27
3rd 5 "	6
4th 5 "	3
5th 5 "	0.6
Total	88.5
15 ml eluate, zeolite column (a)	84
15 " " " (b)	81
15 " " " (c)	86
15 " " " (d)	86

\* The fluorescence was compared with a pure solution standard made up directly in 25 ml. of 25 per cent potassium chloride without passage through the column.

† Solid potassium chloride was added to these solutions to 25 per cent concentration before the alkalinization.

analysis 23 mg. of nicotinic acid (predominantly as the amide) was ingested. The excretion values before and after postprandial dosage with 50 mg. of nicotinamide in aqueous solution are given. The total excretions following dosage were corrected for the basal figures to obtain values for the extra urinary excretions attributable to the test dose. Fairly good correlation was obtained between the basal values and the fractions of the test dose excreted. However, the urinary excretion figures could not be correlated with the size of the individuals.

In Fig 3 are plotted the values for the extra urinary excretions of N<sup>1</sup>-methylnicotinamide for five of the same subjects taking at three widely

TABLE III

Urinary Excretion of N<sup>1</sup>-Methylnicotinamide by Normal Adult Males before and after Supplementation of Basal Ration with 50 Mg. of Nicotinamide

The test dose was taken in aqueous solution immediately after the largest meal of the day. The subjects subsisted during the test periods on a standardized basal ration which furnished 23 mg. of nicotinic acid (predominantly as the amide) per day.

Subject	Height	Weight	Surface area*	24 hr. urinary excretion of N <sup>1</sup> -methylnicotinamide†				
				Subjects on basal ration alone		Subjects receiving supplement + basal ration		
				Basal excretion	Per cent of dietary intake	Total excretion	Extra excretion	Per cent of test dose
	cm.	kg.	sq.m.	mg.		mg.	mg.	
F. O.	161	61	1.65	3.1	13	8.7	5.6	11
D. K.	166	58	1.65	3.4	15	8.7	5.3	11
J. C.	166	63	1.70	8.0	35	22.7	14.7	29
E. M.	166	68	1.76	7.2	31	20.8	13.6	27
D. M.	170	75	1.86	7.7	33	17.5	9.8	20
E. E.	174	73	1.87	5.8	25	10.1	4.3	9
M. H.	174	75	1.89	4.8	21	10.0	5.2	10
H. H.	191	86	2.15	3.8	17	11.5	7.7	15
Average..	171	70	1.82	5.5	24	13.8	8.3	17

\* Estimated directly from a nomogram (18) based upon figures for height and weight.

† Calculated as nicotinamide on an equimolar basis.

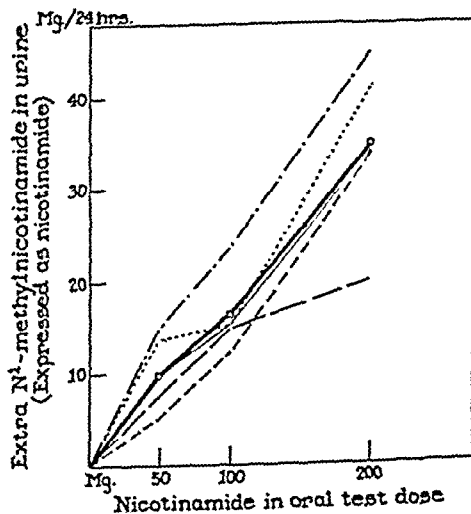


FIG. 3. Urinary excretion of N<sup>1</sup>-methylnicotinamide by normal subjects after postprandial ingestion of nicotinamide in aqueous solution. The heavy line represents the average responses, the fine lines the individual responses.

spaced intervals test doses of 50, 100, and 200 mg. of nicotinamide. The same standardized dietary and dosage procedures mentioned above were employed during each test period. Although there was an appreciable variation between subjects in the values obtained at a given dose level, the responses of each subject were fairly consistent at different levels of nicotinamide intake.

The rate of excretion of extra N<sup>1</sup>-methylnicotinamide following oral dosage with 50 mg. of nicotinamide was also determined with these subjects. The corresponding values for the preceding basal 24 hour period were subtracted from the figures obtained following nicotinamide dosage. The results are presented in Table IV. On the average, 37 per cent of the

TABLE IV

*Periodic Urinary Excretion of Extra N<sup>1</sup>-Methylnicotinamide\* by Normal Adult Males Following Oral Postprandial Dosage with 50 Mg of Nicotinamide*

The test dose was taken at 1 00 p m immediately after a large, noonday meal, and urine samples were collected for the periods indicated

Subject	Extra periodic excretion				Total extra excretion during 24 hrs
	1st 4 hrs	2nd 4 hrs	3rd 4 hrs	Subsequent 12 hrs	
	mg	mg	mg	mg	mg
E M	4 2	2 8	1 3	5 3	13 6
J C	4 2	1 4	1.7	7 4	14 7
D M	3 9	1 6	1 3	3 0	9 8
M H	3.5	1 3	0 3	0 1	5 2
H H	3 4	1 9	0 7	1.7	7 7
Average	3 8	1.8	1 1	3 5	10 2

\* Calculated as nicotinamide on an equimolar basis.

total extra excretion occurred during the first 4 hour period. The remaining 63 per cent was almost equally partitioned during the subsequent 20 hour collection period. These results are similar to those recently reported by Ellinger and Coulson (20). It is worth pointing out that the rate of excretion of N<sup>1</sup>-methylnicotinamide following oral dosage with nicotinamide is comparable to that for the other water-soluble vitamins, thiamine, ascorbic acid, and riboflavin (13), though the latter appear in the urine unchanged. These studies were conducted simultaneously with the same subjects.

#### DISCUSSION

The procedure for the determination of N<sup>1</sup>-methylnicotinamide described in the present report has many advantages over others previously pub-

lished. The use of the semiquantitative colorimetric procedure requires rigid dietary control (3, 6) in order to limit the intake of trigonelline, which is ubiquitous in nature. The colorimetric method fails to differentiate the nicotinamide metabolite from trigonelline, a normal urinary constituent passively excreted following ingestion of the betaine (2, 4). The early fluorometric method (8) lacked a crystalline standard for recovery tests, and could only be regarded as semiquantitative. The use of a quinine sulfate solution as a primary standard (8) has been found unsatisfactory in our hands compared with crystalline N<sup>1</sup>-methylnicotinamide. The errors may have been due to variations in temperature and concentration of dissolved gases (21) in the quinine solution. The importance of limiting the size of the urine aliquot taken for adsorption on the zeolite has also been recognized recently by Coulson and associates (22). Procedures for testing larger aliquots (11, 12) undoubtedly give low results. It is probably for these reasons and because of the small test dose (10 mg.) of nicotinic acid that Mickelsen (23) failed to find any increased urinary excretion of N<sup>1</sup>-methylnicotinamide following dosage with nicotinic acid. Furthermore the amount of the metabolite found in the urine following nicotinic acid dosage may be only one-half of that associated with nicotinamide (20) owing to the fact that nicotinic acid is also excreted to an appreciable extent as nicotinuric acid (2).

In the recent paper by Ellinger and Coulson (20) much emphasis is directed to the disproportionately large basal excretions of N<sup>1</sup>-methylnicotinamide. The authors offer this as evidence for the availability to the organism of the vitamin from a source other than dietary. Unfortunately their basal rations were not tested for nicotinamide content. In the present study when such analyses were made the percentage excretion of the dietary vitamin as N<sup>1</sup>-methylnicotinamide was found to approximate more closely that following dosage with extra nicotinamide. Nevertheless, the somewhat greater percentage excretions obtained during the basal period must be regarded as real, since without exception these were observed with all eight test subjects. However, before the hypothesis of Ellinger and Coulson (20) can be accepted, the absolute specificity of the fluorometric method for N<sup>1</sup>-methylnicotinamide in urine must be demonstrated. No improvement in the specificity of the present method occurs when preliminary charcoal clarification of the sample, as described by Najjar (11), is carried out.

#### SUMMARY

A method is presented for the chemical determination of N<sup>1</sup>-methylnicotinamide in urine. The procedure involves direct adsorption of the metabolite on an activated zeolite column, elution with potassium chloride,

treatment of the eluate with alkali, development of the fluorescence to maximal intensity in butanol, and comparison of the butanol solution in a fluorometer with a standard similarly treated. Correction is made for the blank fluorescence prior to alkalization. Quantitative recovery of the added N<sup>1</sup>-methylnicotinamide chloride is routinely obtained only when the urine aliquot taken for test is a 6 minute sample or less. The procedure has been simplified so that only one zeolite column is necessary and fifteen urine samples may be tested by a single analyst in one day.

While subsisting on an adequate diet containing 23 mg. of nicotinic acid (predominantly as the amide) per day, eight normal subjects excreted in 24 hours from 3.1 to 8.0 mg. of N<sup>1</sup>-methylnicotinamide, calculated as nicotinamide. The average basal excretion value was 24 per cent of the dietary intake. The extra excretion of the metabolite following oral postprandial dosage with 50 to 200 mg. of nicotinamide was approximately 20 per cent. Although there was an appreciable variation in the values obtained at a given dose level, the responses of each subject were fairly consistent at different levels of intake. The rapid rate of excretion of the metabolite following dosage with nicotinamide is comparable to that for thiamine, ascorbic acid, and riboflavin.

The authors are indebted to Mr. Sidney Weiss of these laboratories for the preparation of the N<sup>1</sup>-methylnicotinamide chloride used as the standard in these studies.

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# A NOTE ON THE AMINO ACID COMPOSITION OF TYROCIDINE

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Several details of the amino acid composition of the crystalline bacterial polypeptide tyrocidine are considered in this communication: (a) the presence of *l*(+)-aspartic acid has been established by isolation; (b) alanine has been shown not to be present in significant amounts; (c) the presence of *l*(+)-valine and *l*(+)-leucine has been confirmed; (d) evidence has been obtained that the tryptophane present is of the *l*(+) configuration.

While indirect evidence has suggested that aspartic acid is present in tyrocidine hydrolysates (1, 2), this has not heretofore been established. Alanine, upon the basis of the amino nitrogen precipitated by dioxypyridic acid, was supposed to be present (3), but this amino acid could not be detected by Gordon, Martin, and Synge (2). These latter authors have isolated a preparation of acetylvaline from tyrocidine, but the physical constants and analyses observed for this preparation make desirable a confirmation of the presence of *l*(+)-valine. Finally, no evidence has been reported as to which stereoisomeric form of tryptophane is present.

## EXPERIMENTAL

*Isolation and Determination of l-Aspartic Acid*—Tyrocidine<sup>1</sup> was hydrolyzed under a carbon dioxide atmosphere in a sealed tube, 24 hours at 110°, in a solution of 1 volume of acetic acid and 2 volumes of 6 N hydrochloric acid. The barium salts of the dicarboxylic amino acids were separated (5) and from this fraction the copper salts insoluble in water were obtained (6). Upon the basis of the nitrogen content at this point in four experiments, aspartic acid was estimated to represent 5.3, 5.6, 5.0, and 4.6 per cent of the nitrogen of tyrocidine. This fraction was benzoylated according to Fischer (7); benzoyl-*l*(+)-aspartic acid was separated and recrystallized twice from water. The needles melted at 179°, uncorrected, the same melting point shown by synthetic benzoyl-*l*(+)-aspartic acid. No depression of melting temperature occurred upon mixing the two. The yield from 100 mg. of tyrocidine hydrochloride was, after recrystallizations, 3 mg.

<sup>1</sup> Tyrocidine hydrochloride was isolated according to Hotchkiss and Dubos (4) from tyrothricin furnished by the Wallerstein Company, Inc.



*Absence of Alanine. Source of Interference with Method of Bergmann and Niemann (8)*—Measurement of the acetaldehyde produced by ninhydrin according to the method of Alexander<sup>2</sup> gave values for alanine representing only about 0.6 per cent of the nitrogen of tyrocidine, both for a preparation previously studied (3) and for recent preparations. Under the conditions we employed, leucine caused some interference and probably accounted for much of this apparent alanine recovery. Unless the tyrocidine molecule is much larger than the evidence indicates (1), these results are to be interpreted to indicate that alanine is probably not present in the tyrocidine molecule.

The precipitate obtained from tyrocidine hydrolysates by the addition of dioxypyridic acid has been found to be a tryptophane salt. *l*(+)-Tryptophane showed a solubility of 3 mg. per ml. at 5° in a saturated solution of sodium dioxypyridate in normal hydrochloric acid. This interference by tryptophane undoubtedly accounts for the high values for alanine in gramicidin obtained by the same reagent (3). Alexander's method has given values essentially confirming those of Gordon, Martin, and Syge (9) for alanine in gramicidin.<sup>3</sup>

*Bacterial assays for l(+)-valine, l(+)-leucine, and l(+)-tryptophane* were made with *Lactobacillus arabinosus* according to the method of Hegsted (10). Each value obtained was based upon assay at five levels. *l*(+)-Valine values corresponded to 7.1, 8.5, and 7.1 per cent of the nitrogen, and *l*(+)-leucine to 8.4, 7.8, and 8.5 per cent of the nitrogen. Of three acid hydrolysates tested, two showed low recoveries of *l*(+)-tryptophane, indicating extensive destruction, but the *l*(+)-tryptophane recovered by bacterial assay from a third hydrolysate represented 8.1 per cent of the nitrogen of tyrocidine. The latter value corresponded to a value of 8.5 per cent of tryptophane found for this same hydrolysate by the chemical method of Bates (11). Thus, most of the tryptophane which survived acid hydrolysis appeared to be *l*(+)-tryptophane. The characteristic instability of tryptophane during the acid hydrolysis of tyrocidine, in contrast to the constancy of the recovery of tryptophane in the case of gramicidin, has been observed also by Gordon, Martin, and Syge (2). The values found for *l*(+)-valine and *l*(+)-leucine are similar to those of Gordon, Martin, and Syge.

#### SUMMARY

The presence of *l*(+)-aspartic acid in tyrocidine hydrolysates has been established by isolation and identification. Alanine has been shown to be present in small amounts if at all. The presence of *l*(+)-valine has been

<sup>2</sup> Alexander, B., personal communication. Dr. Alexander has kindly furnished us with directions for this determination in advance of publication.

<sup>3</sup> Christensen, H. N. and Hegsted, D. M., unpublished results.

confirmed and evidence obtained that the tryptophane is of the *l*(+) configuration. The following analytical results have been obtained, expressed as per cent of the tyrocidine nitrogen: aspartic acid 5.1, *l*(+)-valine 7.6, *l*(+)-leucine 8.2.

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# THE FAT, WATER, CHLORIDE, TOTAL NITROGEN, AND COLLAGEN NITROGEN CONTENT IN THE TENDONS OF THE DOG

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This report includes analytical data on the right and left tendons of Achilles and the tendons of the flexor and extensor muscles of the front legs of dogs. The usage of these data for a satisfactory estimation of connective tissue in a tissue is based on the work of Manery, Danielson, and Hastings (1), who first suggested that in a tissue the fibers are surrounded by an extracellular phase comprising the vascular system, along with blood and connective tissue mixed with an ultrafiltrate of plasma. For defining the values for the amount of connective tissue in an organ, the composition of tendon must be known, the assumption being made that the ratio of collagen nitrogen to total nitrogen is the same in connective tissue as in tendon. Making this assumption, Manery *et al.* studied the water and the electrolyte content of the serum and tendons of rabbits. Later, Muntwyler, Mellors, Mautz, and Mangun (2) made a similar study on the serum and tendons of dogs. Neither of these two studies included the ratio of collagen nitrogen to the total nitrogen in the tendon, but instead the values of beef tendon as determined by Mitchell, Zimmerman, and Hamilton in 1926 (3) were used.

The aim of this study, therefore, is to define values for water, chloride, fat, total nitrogen, and collagen nitrogen in the tendons of Achilles and the tendons of the flexor and extensor muscles of the dog. The report gives data on a group of fourteen normal dogs.

## *Methods and Procedures*

Dogs weighing 15 to 18 kilos, under nembutal anesthesia, were used for the removal of the tendons. Blood was taken under oil from the femoral artery for the serum analysis. The tendons of Achilles and the tendons of the flexor and extensor muscles of the front legs were removed and placed in glass-stoppered weighing bottles. The sections of the tendons removed were the bands between the bony insertion and their union with the muscles. The available lengths of the tendons from the flexor and extensor muscles were used. The Achilles tendons were prepared for analysis as follows: The connective tissue sheath covering the tendons of the three heads was



# STUDIES ON ORGANIC FACTORS REQUIRED FOR PREVENTION OF ANEMIA IN CHICKS\*

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The first evidence for the existence of an organic factor specific for the prevention of anemia in chicks was reported in 1940. In that year, Hogan and Parrott (1) reported the discovery of a factor in liver, designated vitamin B<sub>c</sub>, which prevented the anemia that develops in chicks fed a simplified diet.

Hutchings, Bohonos, Hegsted, Elvehjem, and Peterson (2), in 1941, reported that certain liver fractions which are rich in the "norit eluate factor," necessary for growth of *Lactobacillus casei* (3, 4), were active in promoting growth in chicks fed a purified diet. Later, Mills, Briggs, Elvehjem, and Hart (5) found that similar fractions, containing the *Lactobacillus casei* factor, were required for hemoglobin formation as well as for growth and feather development.

Pfiffner and associates (6), in 1943, reported the isolation from liver of a yellow crystalline acid which was highly active in promoting growth of *Lactobacillus casei* and *Streptococcus lactis* R and which prevented anemia in chicks fed a purified diet. They concluded that this substance is identical with vitamin B<sub>c</sub> of Hogan and Parrott (1) and probably identical with Peterson's "eluate factor" (3) and Williams' folic acid (7).

In 1943 Stokstad (8) reported the isolation from both liver and yeast of a highly purified crystalline factor necessary for *Lactobacillus casei*. Chemical analyses of the compound from liver agreed well with those reported for vitamin B<sub>c</sub> by Pfiffner and associates (6) and by Binkley and associates (9). Stokstad (8) believed his crystalline *Lactobacillus casei* factor from liver to be identical with vitamin B<sub>c</sub>.

Hill, Norris, and Heuser (10), using a purified diet, showed that neither factor R nor factor S of Schumacher and coworkers (11) was identical with folic acid. They found that at least one other factor aside from folic acid is required for the prevention of anemia in chicks, and that folic acid, if anemia-preventing, is active only in the presence of the second factor.

O'Dell and Hogan (12) reported that the substitution of a biotin concentrate for a liver extract in the basal diet increased the incidence of

\* This work was supported in part by the establishment of a fellowship at Cornell University by the Western Condensing Company, San Francisco, California.

anemia but, in this case, the chicks failed to respond as well to treatment with vitamin B<sub>12</sub>. They suggested that these difficulties may have been due to a lack of some other limiting factor.

Recently Scott and associates (13) reported that hydrogen peroxide-treated pyridoxine and the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine promoted growth and prevented anemia in chicks fed a purified diet, supplemented with factor S of Schumacher and associates (11) which was prepared from strain S, dried brewers' yeast.<sup>1</sup>

Later, the lactone of 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine was synthesized here,<sup>2</sup> and an experiment was undertaken to compare its antianemic activity with that of the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine. Anemia, however, was not completely prevented as in the earlier work, although the lots receiving the lactones showed some improvement in hemoglobin formation over the controls. The factor S concentrate used in this experiment was prepared from a different supply of strain S, dried brewers' yeast, than that used in the earlier studies.

From the work of Pfiffner and associates (6), O'Dell and Hogan (12), Stokstad (8), and Hill and coworkers (10) it seemed logical to suspect that the *Lactobacillus casei* factor (vitamin B<sub>12</sub>) is required in addition to the lactone for the complete prevention of anemia, and that the factor S concentrate used in the earlier study (13) contained more of the *L. casei* factor than did that made from the new yeast. Microbiological assays of the two diets with *L. casei* showed that the diet containing factor S prepared from the first sample of yeast contained 2½ times as much *L. casei* factor as the diet containing factor S prepared from the new yeast.

These findings led to the studies described in this report in which evidence is presented demonstrating that both the *Lactobacillus casei* factor and one of the lactones are required for the complete prevention of anemia in chicks fed a purified diet.

In view of the discovery that the lactones possess vitamin activity, as shown by the data reported in the earlier communication (13) and that presented here, we have given the name  $\alpha$ -pyracin to the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine and the name  $\beta$ -pyracin to the isomeric 4-carboxy lactone.

#### EXPERIMENTAL

The studies reported in this paper were conducted with white Leghorn chicks, fifteen chicks per lot. The chicks were fed a purified diet

<sup>1</sup> We are indebted to Anheuser-Busch, Inc., St. Louis, Missouri, for the strain S, dried brewers' yeast.

<sup>2</sup> Scott, M. L., Norris, L. C., Heuser, G. F., and Bruce, W. F., *J. Am. Chem. Soc.*, 67, 157 (1945).

similar in composition to that of Hill, Norris, and Heuser (10). The composition of the basal diet is presented in Table I.

In order to assure that the supplements under investigation were acting primarily upon the chicks themselves and not upon the intestinal bacteria, *p*-aminobenzoic acid was omitted from the basal diet and 1 per cent of succinylsulfathiazole (sulfasuxidine) was added. When this diet was fed alone, growth failure and severe anemia occurred within the first 3 weeks, and the mortality was usually extreme. Comparisons of chicks receiving this diet with those receiving the diet without sulfasuxidine showed that the sulfonamide had no detrimental effect upon the chicks.

TABLE I  
*Composition of Basal Diet*

Ingredient		Ingredient	
	gm		mg.
Corn-starch	50.7	Nicotinic acid	3.0
Purified casein	27.5	Calcium <i>d</i> -pantothenate	1.63
Gelatin	7.5	2-Methyl-1,4-naphthoquinone	1.0
Salt mixture*	5.0	Riboflavin	1.0
Soy bean oil†	4.5	$\alpha$ -Tocopherol	1.0
Cellophane	3.0	Pyridoxine hydrochloride	0.5
Succinylsulfathiazole	1.0	Thiamine chloride	0.5
Fish liver oil‡	0.5	Biotin	0.01
Choline chloride	0.2		
Inositol	0.1		

\* Described by Hill, Norris, and Heuser (10)

† Phosphoric acid added to the extent of 0.2 per cent of soy bean oil as an antioxidant.

‡ Contained 400 Association of Official Agricultural Chemists units of vitamin D and 2000 U. S. P. units of vitamin A per gm

Immediately after the preparation of the experimental diets by the addition of the various supplements to the basal diet, the experimental diets were placed in a refrigerator at 4.4°. Only enough of the diets was withdrawn at a time to supply the chicks the following 24 hours.

*Experiment 1*—This experiment was conducted to show whether or not the *Lactobacillus casei* factor<sup>3</sup> supplements the pyracins in antianemic and growth activity, and whether or not factor S is required in addition to these factors for the prevention of anemia. All of the supplements were included at a level of 50  $\gamma$  per 100 gm. of diet except factor S, which was

<sup>3</sup> We are indebted to Dr. E. L. R. Stokstad and Dr. B. L. Hutchings of the Lederle Laboratories, Inc., Pearl River, New York, for a sample of crystalline *Lactobacillus casei* factor.



added in an amount equivalent to 5 per cent of original yeast. A lot of chicks receiving a commercial chick diet was included in this study as a positive control. The results of this experiment are presented in Table II, Experiment 1.

The results demonstrated that, when fed alone, neither  $\alpha$ - nor  $\beta$ -pyracin nor the *Lactobacillus casei* factor was effective in promoting growth or preventing anemia. The high mortality occurring on the basal diet, how-

TABLE II  
Results of Growth and Hemoglobin Studies

Experi- ment No.	Supplement*	No. of surviving chicks,† 3 wks.	Average weight, 3 wks. gm.	Hemoglobin, 3 wks. gm. per 100 cc.
1	None	10	79.9	4.17
	$\alpha$ -Pyracin	9	89.9	4.30
	$\beta$ -Pyracin	9	99.5	4.00
	<i>Lactobacillus casei</i> factor (LCF)	13	96.3	6.50
	Factor S concentrate (FSC)	8	75.0	5.80
	$\alpha$ -Pyracin + LCF	14	120.7	8.95
	" + " + FSC	13	120.3	9.44
	$\beta$ -Pyracin + "	14	144.3	9.53
	" + " + FSC	12	146.0	9.82
2	Commercial chick diet	15	191.9	10.10
	None	14	93.2	5.16
	$\beta$ -Pyracin	15	91.0	6.70
	<i>Lactobacillus casei</i> factor (LCF)	15	105.3	6.61
	Pyridoxal	15	97.5	6.14
	$\beta$ -Pyracin + LCF	15	105.0	9.50
	Pyridoxal + "	14	94.8	7.11

\* All supplements in Experiment 1 were added at a level of 50  $\gamma$  per 100 gm. of diet except factor S concentrate, which was added equivalent to 5 per cent of original yeast. In Experiment 2 all supplements were added at a level of 25  $\gamma$  per 100 gm. of diet.

† All lots were started with fifteen white Leghorn chicks of mixed sexes.

ever, was largely prevented when the *L. casei* factor was added to it. The combination of either  $\alpha$ - or  $\beta$ -pyracin with the *L. casei* factor produced a marked gain in weight over the controls and was completely effective in the prevention of anemia. This study also demonstrated that, in the presence of the *L. casei* factor,  $\beta$ -pyracin was considerably more effective in promoting growth at the 50  $\gamma$  level than  $\alpha$ -pyracin, but was only slightly more effective in preventing anemia. In the presence of the *L. casei* factor the addition of factor S had little effect.

The chicks fed the practical commercial chick diet showed an appreciable

gain in weight over those receiving the experimental diet supplemented with  $\beta$ -pyracin and *Lactobacillus casei* factor. This may be explained by the fact that the latter diet did not contain factor R of Schumacher and coworkers (11).

*Experiment 2*—This experiment was conducted to determine whether or not lower levels of  $\beta$ -pyracin and the *Lactobacillus casei* factor prevent anemia without affecting growth. The results of this experiment are presented in Table II, Experiment 2.

The results showed that  $\beta$ -pyracin and the *Lactobacillus casei* factor, each at 25  $\gamma$  per 100 gm. of diet, were effective in preventing anemia when supplied together. The slight increase in weight over the controls, however, was of doubtful significance. The combination of these factors increased the hemoglobin level almost 3 gm. per 100 cc. of blood above that of the lots receiving  $\beta$ -pyracin or *L. casei* factor alone, while the average weight of the chicks receiving both factors was no better than that of the chicks receiving *L. casei* factor alone. The results of this experiment showed that smaller quantities of the factors are required to prevent anemia than are required to promote growth.

Snell (14) has recently reported a new factor, pyridoxal, which is active in promoting growth of *Lactobacillus casei* when added to the culture medium in place of pyridoxine. In view of its similarity in structure to  $\beta$ -pyracin and its growth-promoting activity for *L. casei*, pyridoxal<sup>4</sup> was fed to two lots of chicks in this experiment. One lot received the basal diet supplemented with 25  $\gamma$  of pyridoxal per 100 gm.; the other received both pyridoxal and *L. casei* factor at this level. A comparison of the antianemic activities of pyridoxal and  $\beta$ -pyracin is presented also in Table II, Experiment 2.

It is evident from these results that pyridoxal was not effective in preventing anemia when supplied together with the *Lactobacillus casei* factor, although a slight improvement in hemoglobin level was obtained. This may have been due to oxidation, in part, to  $\beta$ -pyracin, since this aldehyde appears to be very susceptible to oxidation.

Under certain conditions it is possible that even pyridoxine, a less readily oxidizable compound than pyridoxal, is converted to a very slight extent to a pyracin. O'Dell and Hogan (12) reported that the incidence of anemia in chicks on their diet was less at the higher levels of pyridoxine. Under our conditions, however, no conversion apparently occurred, since increasing the level of pyridoxine from 500  $\gamma$  per 100 gm., the amount in the basal diet, to 1000  $\gamma$  was ineffective in producing any increase in hemoglobin formation.

<sup>4</sup> We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the pyridoxal.

*Hematological Studies*—Hematological studies were conducted to determine the type of anemia which develops in chicks receiving the basal diet and to ascertain the changes in the blood picture produced by the administration of  $\beta$ -pyracin and the *Lactobacillus casei* factor, singly and combined. Red blood cell counts, hematocrit, and hemoglobin determinations were made simultaneously on at least five chicks from each lot studied. From these values the average individual cell volumes, the average amounts of hemoglobin per cell, and the average hemoglobin per 100 cc. of packed cells were calculated. Representative results of these studies are presented in Table III.

The average individual cell volume was obtained by dividing the hematocrit value by the number of erythrocytes and converting this value to

TABLE III  
Results of Hematological Studies

Supplement	Red blood cells per c mm.	Hematocrit	Hemoglobin	Average individual cell volume	Average amount hemoglobin per cell	Average hemoglobin per 100 cc packed cells
	millions	per cent	gm. per 100 cc.	cubic microns	micro-micrograms	per cent
None ..	0.858	11.2	2.8	132.2	38.7	25.4
$\beta$ -Pyracin	1.520	20.1	7.4	134.6	52.4	38.9
<i>Lactobacillus casei</i> factor (LCF)	1.912	23.6	6.5	125.4	34.4	27.7
$\beta$ -Pyracin + LCF	2.192	26.0	9.8	129.0	46.2	38.1
Commercial chick diet	2.214	26.7	10.0	122.1	47.9	38.9

cubic microns. The average amount of hemoglobin per cell was determined by dividing the gm. of hemoglobin per 100 cc. of blood by the number of erythrocytes per 100 cc. This value is expressed in micromicrograms of hemoglobin per cell. The average hemoglobin per 100 cc. of packed cells was determined by dividing the gm. of hemoglobin in 100 cc. of blood by the hematocrit value. This value is the one used in determining the relative pigment content of the erythrocytes (15).

The kind of anemia which developed in chicks on the basal diet was the macrocytic, hypochromic type, since the average individual cell volume was larger and the average amount of hemoglobin per 100 cc. of packed cells was smaller than the corresponding values for the chicks on the commercial diet.

Adding  $\beta$ -pyracin alone to the basal diet resulted in marked increases in both the red blood cell count and the hematocrit values, amounting to

almost twice the values obtained on the chicks receiving the basal diet only. The *Lactobacillus casei* factor added alone also improved these values markedly. Although both  $\beta$ -pyracin and the *Lactobacillus casei* factor, when fed singly, produced an increase in the hemoglobin level over the controls, neither was completely effective in preventing anemia.

The addition of the *Lactobacillus casei* factor alone to the diet resulted in a lowering of the average individual cell volume, thus changing the type of anemia to a normocytic, hypochromic anemia. On the other hand, supplementing the diet with  $\beta$ -pyracin alone did not affect the cell size, but raised the value for the average amount of hemoglobin per 100 cc. of packed cells to normal, thereby changing the kind of anemia to the macrocytic, normochromic type. The addition of both the *Lactobacillus casei* factor and  $\beta$ -pyracin prevented the anemia completely, giving a blood picture corresponding to that observed in the chicks receiving a practical chick diet.

Hogan and Parrott (1), using a basal diet containing a 95 per cent alcohol extract of liver, reported that the size of the red blood cells and the amount of hemoglobin per cell in chicks were increased above normal. These values correspond to those obtained by us when  $\beta$ -pyracin alone was added to the diet. In later work, O'Dell and Hogan (12) removed the liver extract from their diet and obtained a macrocytic, hypochromic anemia corresponding to the type of anemia which we obtained on our basal diet.

From the data of Campbell, Brown, and Emmett (16, 17) calculation of average individual cell volumes showed that the size of the cell decreased and approached normal as the level of vitamin B<sub>6</sub> was increased. This is in agreement with the results reported here when *Lactobacillus casei* factor alone was added to the diet.

#### SUMMARY

Evidence has been presented which demonstrates that the *Lactobacillus casei* factor and either the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine or the lactone of 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine are required for the complete prevention of the macrocytic, hypochromic anemia that develops in chicks fed a purified diet. The 5-carboxy lactone has been designated  $\alpha$ -pyracin and the isomeric 4-carboxy lactone  $\beta$ -pyracin.

$\beta$ -Pyracin was found to be considerably more active in promoting growth than  $\alpha$ -pyracin, but was only slightly more effective in preventing anemia. Smaller quantities of  $\beta$ -pyracin and the *Lactobacillus casei* factor were required to prevent anemia than were required to promote growth.

The results of hematological studies showed that when the *Lactobacillus casei* factor alone was added to the diet, the kind of anemia which developed

was a normocytic, hypochromic type. When  $\beta$ -pyracin was added alone, the kind of anemia that occurred was a macrocytic, normochromic type.

The technical assistance of J. T. McHenry and C. L. Schenholm is gratefully acknowledged.

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# THE DETERMINATION OF THE ALBUMIN AND GLOBULIN CONTENTS OF HUMAN SERUM BY METHANOL PRECIPITATION

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Although the determination of the albumin and globulin contents of human serum by neutral salt fractionation, as in Howe's method (1), has proved useful because of its simplicity, such methods have definite limitations. Butler (2) has shown that the albumin-globulin separation by neutral salt precipitation is not sharp and that these fractions overlap one another grossly. Leutscher (3) has also pointed out that salting-out methods are most inaccurate in cases in which they are of greatest clinical interest, and that a change in the technique of the salting-out methods is indicated.

While it is impossible to defend any of these techniques as necessarily yielding absolute albumin and globulin values, accumulated evidence indicates that electrophoretic data have more meaning than have results obtained by any salting-out method. The method described in the present paper has been found to yield albumin and globulin values which check closely with the results obtained by the electrophoretic method.

The method is based on the observation of the present authors that normal human serum may be satisfactorily separated into its globulin and albumin components by appropriate treatment with methanol. Electrophoretic analyses of whole serum, and of the separated albumin and globulin fractions, showed that at 0° almost all of the serum albumin remains soluble at a concentration of 42.5 per cent methanol, in the pH range 6.7 to 6.9, and at ionic strength of about 0.03, while the globulins are almost quantitatively precipitated.

A comparison of the albumin-globulin ratios obtained electrophoretically and by the methanol technique as well as by sodium sulfate separation is given in Table I and indicates that the results obtained with the first two methods agree within 5 per cent for normal serums, and within 5 to 10 per cent for abnormal serums. The results by the sodium sulfate method are in far less satisfactory agreement with the electrophoretic analysis, confirming the recent observation of Dole (4) that albumin-globulin ratios, measured electrophoretically, are roughly two-thirds the ratio found by chemical fractionation.

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*Procedure**Reagents—*

*Methanol reagent.* 607 ml. of c.p. methanol are added with mixing to 393 ml. of distilled water. The mixture is cooled to 0°, and finally made up to 1 liter with cold methanol.

*Acetate buffer.* 72 ml. of 1 M acetic acid and 12 ml. of 1 M NaOH are diluted to 1 liter with distilled water.

TABLE I  
*Comparison of Albumin-Globulin Ratio*

Serum No.	Albumin, gm. per 100 ml.			Globulin, gm. per 100 ml.			Albumin-globulin ratio		
	Electrophoretic	Methanol	Na <sub>2</sub> SO <sub>4</sub>	Electrophoretic	Methanol	Na <sub>2</sub> SO <sub>4</sub>	Electrophoretic	Methanol	Na <sub>2</sub> SO <sub>4</sub>
1	3.29	3.55	4.53	4.96	4.70	3.72	0.65	0.75	1.2
2	3.29	3.34	5.08	4.07	4.02	2.28	0.83	0.85	2.3
3	4.0	4.13	5.04	4.37	4.24	3.33	0.93	1.0	1.5
4	3.64	3.79	4.63	4.79	4.64	3.80	0.76	0.82	1.2
5	3.2	3.4	4.21	3.6	3.4	2.59	0.90	1.0	1.6
6	4.14	4.29	4.75	3.28	3.13	2.67	1.2	1.3	1.8
7	3.49	3.43	4.19	4.13	4.10	3.43	0.84	0.82	1.2
8	3.42	3.62		3.70	3.50		0.93	1.04	
9	3.49	3.68		3.58	3.39		0.96	1.08	
10*	3.98	4.12	4.58	2.66	2.52	2.06	1.5	1.6	2.2
11	4.40	4.41	5.20	2.82	2.81	2.02	1.5	1.5	2.5
12	4.10	4.24	4.22	2.7	2.56	2.58	1.54	1.60	1.6
13	3.95	4.00		3.15	3.1		1.25	1.3	

Electrophoretic analyses were performed by Dr. J. W. Williams, University of Wisconsin; Dr. D. Gordon Sharp, Duke University; Dr. L. G. Longworth, The Rockefeller Institute for Medical Research; Dr. John A. Leutscher, Johns Hopkins Hospital; and at the Department of Physical Chemistry, Harvard Medical School.

\* Serums 10 to 13 from normal adults.

*Method*

The serum and all reagents should be maintained at all times between 0° and +1°. This may be achieved either by working in a 0° cold room or with the aid of a small ice bath, or by the use of a low temperature liquid bath.

2.0 ml. of fresh serum are pipetted into a 15 ml. conical centrifuge tube and 1.0 ml. of acetate buffer is added with stirring, the temperature being maintained as stated above. To this mixture, 7.0 ml. of cold methanol reagent are added with stirring. After being thoroughly mixed, the tube is allowed to stand at 0° for  $\frac{1}{2}$  hour, during which time the globulins will have precipitated, while the albumin remains in solution.

Either of two methods for the removal of the precipitate may be followed, depending upon the equipment at hand. If a refrigerated centrifuge is available, the suspension may be centrifuged at  $0^{\circ}$  to  $+2^{\circ}$  at 3000 R.P.M. for 15 minutes, after which the clear supernatant is decanted. A sample of the supernatant is analyzed for nitrogen; the precipitated proteins are discarded. If such a centrifuge is not available, the following procedure has been found to yield equally satisfactory results.

A small funnel fitted with a fluted No. 42 Whatman filter paper is thoroughly chilled in a cold room or ice box. The cold methanol-protein suspension is then filtered and the clear filtrate used for nitrogen analysis. The actual filtration may be done at room temperature, provided sufficient filtrate for analysis, at least 3 ml., is obtained within 5 to 7 minutes after removal of the material from the cold. In our experience, the temperature of the suspension does not rise above  $7^{\circ}$  during this time, and results so obtained have been satisfactory. Because of the volatility of methanol, aliquots for nitrogen analysis are immediately taken and transferred to digestion flasks.

The albumin filtrates from normal serum usually contain from 1.2 to 1.5 mg. of nitrogen per ml. of filtrate, while abnormal serums have varied from 0.8 to 1.8 mg. of nitrogen per ml. of filtrate.

#### Calculations

$500 \times \text{mg. N per ml. filtrate} = \text{gm. albumin N per 100 ml. serum}$

$\frac{\text{Gm. albumin N per 100 ml. serum}}{\text{Gm. total protein N per 100 ml. serum}} = \% \text{ albumin}$

$\text{Gm. total protein N per 100 ml. serum minus gm. albumin N per 100 ml. serum} = \text{gm. globulin N per 100 ml. serum}$

$100 \% - \% \text{ albumin} = \% \text{ globulin}$

$\frac{\% \text{ Albumin}}{\% \text{ Globulin}} = \text{albumin-globulin ratio}$

#### SUMMARY

A method employing methanol under controlled conditions is presented for the determination of the albumin and globulin contents of human serums. Values obtained by this procedure compare favorably with results obtained electrophoretically.

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# STUDIES ON VITAMINS B<sub>10</sub> AND B<sub>11</sub> AND RELATED SUBSTANCES IN CHICK NUTRITION\*

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In 1943 we reported (1) that chicks required two new water-soluble vitamins designated as vitamin B<sub>10</sub>, necessary for proper feather formation, and vitamin B<sub>11</sub>, required for growth. These vitamins were shown to be distinct from "folic acid" as measured by *Streptococcus lactis* R and *Lactobacillus casei* (2). Later, description was made of some of the properties of vitamins B<sub>10</sub> and B<sub>11</sub> as they existed in relatively crude fractions, and of methods for their partial separation (3). Further work on these vitamins, including progress which has been made on their purification, is presented in this paper.

Since we are now using crystalline vitamin B<sub>c</sub> (4) as our standard for bacterial assays, the term "vitamin B<sub>c</sub> activity" will be used instead of the term "folic acid" to include any substance which will cause growth of *Streptococcus lactis* R and *Lactobacillus casei* on a defined medium (5). It is not possible to tell at present whether growth in the bacterial assay of various crude materials is due to the presence of vitamin B<sub>c</sub> itself or to related substances such as have been reported (6-8) by various workers (see review by Luckey *et al.* (9)). We feel, however, that the term "vitamin B<sub>c</sub> activity" is justified, since recently Williams has pointed out

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(10) that there are several different "folic acids," whereas vitamin B<sub>c</sub> is a crystalline product and a suitable standard.

#### EXPERIMENTAL

The experimental conditions for rearing the white Leghorn chicks and methods of assay for vitamins B<sub>10</sub> and B<sub>11</sub> have been described previously (1, 3). The basal ration (No. 494K) is very similar to that used in earlier work and has the following percentage composition: dextrin 61, casein (Smaco vitamin test) 18, gelatin 10, Salts 5 (1) 6, soy bean oil 5, and l(-)-cystine 0.3. Each 100 gm. also contain thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., Ca pantothenate 2 mg., choline chloride 150 mg., nicotinic acid 5 mg., pyridoxine hydrochloride 0.4 mg., biotin 0.02 mg., *i*-inositol 100 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., and  $\alpha$ -tocopherol 0.3 mg. In addition each chick received 1 drop per week of an oil mixture which contains approximately 1200 U. S. P. units of vitamin A and 120 A. O. A. C. units of vitamin D<sub>3</sub>.

Normal growth, feathering, and hemoglobin formation are not attained by chicks fed the basal ration unless a source of unknown vitamins is supplied. The ration does not contain added amounts of *p*-aminobenzoic acid or ascorbic acid even though their addition results in improved growth, feathering, and hemoglobin formation (11, 12). The response to these compounds appears to be indirect (11-13), and therefore they are omitted from the basal ration.

Assays for vitamin B<sub>c</sub> activity were performed by the method of Luckey *et al.* (5) with *Streptococcus lactis* R and *Lactobacillus casei*. The values obtained with *Lactobacillus casei* are not included in this report, since they were similar to those obtained with the other organism. Pure vitamin B<sub>c</sub> has an approximate potency of 80,000, and therefore figures for "folic acid" in our previous papers, based on a potency of 40,000, should be divided by 2 in order to make comparisons with the figures given in this report for vitamin B<sub>c</sub> activity. Each fraction has been assayed after acid treatment by autoclaving at pH 4 for 12 hours (14) and unpublished data). This method has been used to obtain maximal bacterial activity in samples in place of taka-diastrase treatment (15). Representative samples were measured for vitamin B<sub>c</sub> activity also by the use of an enzyme described by Mims, Totter, and Day (16). The values obtained were not appreciably higher than those obtained with acid treatment and thus it is felt that most of the vitamin B<sub>c</sub> "conjugate" reported by Binkley *et al.* (17), present in yeast and liver fractions, is being measured. It is interesting that Stokes (18) has also used acid treatment and has attributed the increased response to a liberation of a thymine-like compound.

A scheme is given which has been used successfully for the purification

*Scheme for Purification of Vitamins B<sub>10</sub> and B<sub>11</sub>*

Super Filtrol eluate (see text)

Make to 75 % ethanol, pH 7

Make to pH 3

Preparation 30 (1)\*  
(some vitamin B<sub>10</sub> and B<sub>11</sub>,  
low vitamin B<sub>6</sub> activity)

Preparation 52A (3)\* (rich  
in vitamin B<sub>10</sub>, B<sub>11</sub>,  
and B<sub>6</sub> activity)

Preparation 214B  
(low activity)

Concentrate and neutralize

Dialyze through cellophane  
sheeting (du Pont, No. 300-PT)

Preparation 233C (high vitamin B<sub>6</sub>  
activity, some vitamin B<sub>10</sub> and B<sub>11</sub>)

Dialyze through Visking cellulose  
casing (2½ in. diameter used)

Preparation 233E†

Preparation 233D†

Make to pH 3

Chromatogram on Super Filtrol column  
and develop with water at pH 3‡

Upper layer

Bottom layer

Preparation 233G†

Preparation 233H†

Add excess Ba(OH)<sub>2</sub> and make to 50 %  
ethanol (let stand in cold overnight)

Combine filtrates  
Preparation 249C  
(low activity)

Reprecipitate in 50 % ethanol

Remove excess Ba with H<sub>2</sub>SO<sub>4</sub>

Preparation 249B†

Make to pH 3 and chromatogram  
with Super Filtrol and Super-Cel§

Unadsorbed filtrate,  
Preparation 249D  
(low activity)

Elute with water, pH 7

Preparation 249E†  
(high in vitamin B<sub>11</sub>,  
some vitamin B<sub>10</sub>, low  
vitamin B<sub>6</sub> activity)

Elute with 50 %  
ethanol, pH 9  
Preparation 249G  
(low activity)

\* Bibliographic reference number.

† See Table I.

‡ Various pH ranges have been used for developing.

§ A mixture of 1 part of Super Filtrol to 5 parts of Johns-Manville Hyflo Super-Cel; column packed dry.

of vitamins B<sub>10</sub> and B<sub>11</sub>. The Super Filtrol eluate,<sup>1</sup> the starting material, was prepared from solubilized liver by a modification of the method of Hutchings, Bohonos, and Peterson (19) (adapted from the work of Snell and Peterson (20)). Methods for the alcohol precipitations and for the dialysis procedure have been described (1, 3).

TABLE I

*Results of Feeding Various Purified Preparations with Basal Ration 494K\**

Preparation No. and level fed†	Vitamin B <sub>10</sub> activity (feather formation)	Vitamin B <sub>11</sub> activity (weight at 4 wks.)	Vitamin B <sub>12</sub> activity added per 100 gm. ration (after acid treatment). <i>Streptococcus lactis</i> R	Hemoglobin per 100 cc. blood‡	Dry matter in supplement per 100 gm. ration
		gm.	γ	gm.	mg.
No supplement.....	30	135	0	7.3 (20)	0
Super Filtrol eluate ≈ 5% (control) .....	100	252	48	9.0 (20)	100
233D ≈ 10 % .....	25	189	2.9	8.0 (4)	2.9
233E ≈ 10 % .....	75	196	17.0		5.2
233G ≈ 10 % .....	55	157	7.8	8.9 (4)	1.7
233H ≈ 10 % .....	75	197	8.9	9.8 (4)	6.5
249B ≈ 15 % .....	45	222	6.0	8.0 (4)	14.2
249E ≈ 20 % .....	50	240	4.8	8.0 (4)	1.25
223C ≈ 15 %    .....	90	253	9.6	8.6 (4)	6.0

\* Six chicks per group except that the basal and control weights are an average of thirty chicks.

† Expressed as ≈ per cent or gm. of starting material (solubilized liver), which has been concentrated to the amount given in the last column, per 100 gm. of ration.

‡ 0 = very poor; 25 = poor; 50 = fair; 75 = good; 100 = very good.

§ The number of chickens tested is given in parentheses.

|| Similar to Preparation 249E; however, the Ba(OH)<sub>2</sub> precipitation step, as shown on the scheme, was omitted and elution from the chromatogram column was carried out at pH 8.5.

### Results

Results obtained with several of the preparations given in the accompanying scheme are presented in Table I. Preparation 249E supplied only 1.25 mg. of dry matter per 100 gm. of ration and produced nearly maximal growth (with only fair feathering), thus effecting a concentration of vitamin B<sub>11</sub>, the growth factor, of nearly 1600-fold.

<sup>1</sup> The Super Filtrol eluate, norit eluate fraction, and similar fractions have been called "folic acid" concentrates by various workers, which has led to the idea that these fractions contain only "folic acid." It should be pointed out that such fractions as these contain other necessary factors as well (e.g., vitamins B<sub>10</sub> and B<sub>11</sub>) which may be as important, or more so, in the nutrition of animals than "folic acid."

Vitamin B<sub>10</sub> was not as readily concentrated as was vitamin B<sub>11</sub>. Preparations 233E, 233H, and 223C, however, caused the production of good feathers (75 or better) with about 6 mg. of dry matter per 100 gm. of ration in each case. An important point to emphasize is that, although vitamin

TABLE II  
Results of Feeding "Folic Acid" Concentrates and Pure *Lactobacillus casei* Factor

Group No.	Supplement to Basal Ration 494K per 100 gm.	Total No. of chicks	No. dead in 4 wks.	Vitamin B <sub>10</sub> activity (see footnote, Table I)	Vitamin B <sub>11</sub> activity (average weight at 4 wks.)	Hemoglobin per 100 cc. blood*
1	No supplement	22	3	30	gm. 127	gm. 6.9 (16)
2	Control (Super Filtrol eluate $\approx$ 5 gm. solubilized liver)	22	0	100	246	8.9 (16)
3	20 $\gamma$ "folic acid" (potency 60,000)†	5	2	25	144	7.2 (4)
4	30 $\gamma$ "folic acid" (potency 60,000)	6	0	45	171	8.6 (4)
5	50 $\gamma$ "folic acid" (potency 60,000)	5	1	55	189	8.5 (4)
6	5 $\gamma$ <i>Lactobacillus casei</i> factor	3	0	40	138	
7	20 $\gamma$ <i>Lactobacillus casei</i> factor	3	0	55	187	8.3 (3)
8	50 $\gamma$ <i>Lactobacillus casei</i> factor	6	0	60	224	8.6 (4)
9	0.5 gm. sulfasuxidine	3	0	25	90	5.3 (3)
10	As Group 9 + 30 $\gamma$ "folic acid" (potency 60,000)	6	0	30	117	6.6 (4)
11	As Group 9 + 20 $\gamma$ <i>Lactobacillus casei</i> factor	3	0	35	128	7.4 (3)
12	25 gm. yellow corn-meal (in place of dextrin)	12	9	22	107	4.6† (4)
13	As Group 12 + 25 $\gamma$ "folic acid" (potency 27,000)	6	3	25	139	5.5† (3)

\* Number of chicks given in parentheses.

† Concentrate obtained from The University of Texas.

‡ 3 week values.

B<sub>10</sub> appears to be more easily lost during concentration than vitamin B<sub>11</sub>, the properties of the two vitamins must be quite similar or they would not be found together to such a great extent in some of these purified fractions. It will be noted that each active fraction contains some vitamin B<sub>6</sub> activity. Thus, the problem again arises as to whether or not vitamin

B<sub>c</sub> *per se* is required in the diet of the chick. To help to answer this question we have fed samples of concentrated "folic acid" and the new *Lactobacillus casei* factor of Hutchings, Stokstad, Bohonos, and Slobodkin (7) (see Table II) and found, as has been reported for the *Lactobacillus casei* factor, that relatively high amounts of these factors did increase growth and feathering. Normal growth, however, was not attained. The bacterial factor did not appreciably counteract the effects of sulfasuxidine (11) which is further evidence that intestinal synthesis of other factors is involved when compounds with vitamin B<sub>c</sub> activity are fed to chicks on the basal ration without the drug.

In two experiments (Group 12) 25 per cent of yellow corn-meal was fed at the expense of dextrin, which resulted in a marked rise in mortality, as well as poorer growth and lower hemoglobin values than obtained with chicks without the corn. When the "folic acid" concentrate was fed in addition to the corn-meal, growth was slightly increased, while mortality was high and the hemoglobin level of the survivors was poor.

The results given in Tables I and II show that chicks on the basal ration develop an anemia (macrocytic), as we have demonstrated previously with chicks on similar rations (21). Although compounds with vitamin B<sub>c</sub> activity cause an increase in hemoglobin values, we find that at least one other factor is concerned in hemoglobin formation, since fractions low in vitamin B<sub>c</sub> activity raised the hemoglobin values appreciably. Fractions rich in either vitamin B<sub>10</sub> or B<sub>11</sub> did not completely prevent the anemia, which suggests the existence of an unknown factor needed for hemoglobin formation. Since the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine had been reported to be a new chick antianemia factor by Scott *et al.* (22), this substance was tested at a level of 500  $\gamma$  per 100 gm. of ration in two 4 week experiments involving twelve chicks. The results showed no antianemia, growth, or feathering activity for the compound when fed on our ration and, therefore, it is not identical with either vitamin B<sub>10</sub>, vitamin B<sub>11</sub>, or the suggested new hemoglobin factor.

#### DISCUSSION

In the past year Hutchings *et al.* (7) have reported the isolation of a new *Lactobacillus casei* factor from an unknown source and stated that the compound was "active in the nutrition of the chick." Results with this substance (Table II) confirm their work (7, 23) to the extent that growth can be obtained with this compound. Hertz and Sebrell (24) have fed the new *Lactobacillus casei* factor of Hutchings *et al.* to chicks and report that an adequate intake of this factor is essential for the normal metabolism of stilbestrol.

Pfiffner *et al.* (4) stated that normal growth and hemoglobin production

were obtained by feeding crystalline vitamin B<sub>6</sub> to chicks receiving a deficient ration. In a later paper Campbell, Brown, and Emmett (25) reported, with the use of a ration somewhat comparable to ours, that 100  $\gamma$  of vitamin B<sub>6</sub> per 100 gm. of ration were required "to maintain the birds on the normal plane" of growth. They also stated that 40  $\gamma$  of vitamin B<sub>6</sub> gave "complete protection for the hematocrit, red blood cell count, hemoglobin and thrombocytes;" however, their data showed that the hemoglobin level was only 7.0 gm. per 100 ml. of blood as compared to a level of 8.7 gm. for the control ration. More recently the same authors (26) state that crystalline vitamin B<sub>6</sub> "has a positive influence on growth (body weight and feathering) and on the blood cell components" and do not claim that *normal* growth itself was attained. Thus, their results actually agree with ours in so far as other factors beside those with vitamin B<sub>6</sub> activity are needed by chicks.

Campbell *et al.* (26) found that crystalline vitamin B<sub>6</sub> had the same biological effect when given parenterally as when given orally. This finding, the authors state, "tends to suggest that the intestinal flora is very probably not influenced" by vitamin B<sub>6</sub>. It is possible, as they admit, that substances may be reexcreted into the intestine from the tissues, thus influencing intestinal synthesis.

From the work herein presented and from the data in former papers (1, 3) it is clear that normal growth, feather formation, and hemoglobin production may be obtained by feeding much lower levels of vitamin B<sub>6</sub> activity than those stated as optimal by Campbell *et al.* (25). Although some vitamin B<sub>6</sub> activity may be required, it is clear from results shown in Table I that no more than approximately 8  $\gamma$  per 100 gm. of ration (most of this amount is "potential" vitamin B<sub>6</sub> activity) are needed, which includes the 3  $\gamma$  present in the basal ration. It has been shown previously (1, 3) that higher levels of vitamin B<sub>6</sub> activity may be fed in the form of relatively crude liver fractions to chicks receiving the deficient ration without giving any response. It is possible that vitamin B<sub>10</sub> or B<sub>11</sub> may have some activity for *Streptococcus lactis* R and *Lactobacillus casei* which would be undifferentiated from vitamin B<sub>6</sub> activity in the assay and which would account for the value given in Table I.

Hill, Norris, and Heuser (27) have recently published further work on factors R and S with the use of new purified rations. In confirmation of our previous results they could find no evidence that vitamin B<sub>6</sub> activity ("folic acid") was required by the chick unless in small amounts. They also report the presence of an antianemia factor in liver which has since been identified with the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxypyridine (22). The relationship of this new factor R to vitamin B<sub>11</sub> is not clear as yet; however, the new factor R preparations appear to



contain vitamin B<sub>11</sub> and some vitamin B<sub>10</sub>. Since factor S is not readily adsorbed on typical adsorbing agents, it appears that this factor is distinct from vitamin B<sub>10</sub> or B<sub>11</sub> and may be related to the growth factor present in kidney residue (21).

Recently Norris has stated (28) that Scott and associates have found that factor S preparations are contaminated with the *Lactobacillus casei* factor which is required, with the above mentioned lactone, for hemoglobin formation in the chick. From the work we have presented in this paper it appears that still another antianemia factor is present in liver concentrates.

In the past year work with other animals has appeared, such as has been done with the chick, demonstrating the biological activity of unknown factors other than substances having direct vitamin B<sub>c</sub> activity. Totter, Shukers, Kolson, Mims, and Day (29), working with monkeys, have concluded that the distribution of "folic acid" was shown to be different from that of vitamin M. More recently, Mallory, Mims, Totter, and Day (30) have stated that vitamin M is the same as or closely related to vitamin B<sub>c</sub> (and related factors) and substances enzymatically convertible to vitamin B<sub>c</sub>. This would distinguish vitamin B<sub>10</sub> and B<sub>11</sub> from vitamin M (see Table I). Welch and Wright (31) have reported the possible existence of a material similar to "folic acid" but inactive for *Streptococcus lactis* R with vitamin activity for the rat. Woolley and Sprince (32) have identified "folic acid" as one of the unknown dietary essentials for the guinea pig; however, they reported that solubilized liver contained other essential factors. This has also been found in our laboratory.<sup>2</sup> Richardson, Chiang, Hogan, and Kempster (33) have reported that only six out of eleven turkey poults on a synthetic ration responded to supplements of vitamin B<sub>c</sub> (indicating other factors were lacking). In work with chicks Binkley *et al.* (17) have confirmed our previous work that chick growth (and hemoglobin formation) did not always correlate with the activity of the supplement as measured with *Streptococcus lactis* R or *Lactobacillus casei*. Whether or not any of these factors required in addition to vitamin B<sub>c</sub> activity are identical with vitamins B<sub>10</sub> or B<sub>11</sub> has not been indicated. It is interesting in this connection that Golberg, De Meillon, and Lavoipierre (34) have recently reported that mosquito larvae require a source of unknown factors which contained vitamins B<sub>c</sub>, B<sub>10</sub>, and B<sub>11</sub> for proper growth, pupation, and pigmentation.

The experiments in which corn-meal was added to our basal ration were carried out because Cunha, Kirkwood, Phillips, and Bohstedt (35) had reported that rats on a basal ration composed largely of ground yellow corn received an insufficient supply of the norit eluate fraction (a source of vitamins B<sub>10</sub> and B<sub>11</sub>). Such a deficiency ordinarily can be produced

<sup>2</sup> Cannon, M. D., Zepplin, M., Elvehjem, C. A., and Hart, E. B., *in press*.

only in rats receiving a synthetic ration with a sulfa drug (36), which indicated to us that corn-meal, in some manner, likewise decreases intestinal synthesis of the unknown factors. This was found to be true with the chick. Growth, mortality, and hemoglobin formation of chicks on the basal ration were appreciably poorer when corn-meal was fed in place of dextrin.

From our previous work and from the work of Pfiffner *et al.* (4), Stokstad (6), Hutchings *et al.* (7), Keresztesy *et al.* (8), and Williams and associates (10, 37), it is evident that what was first believed to be one active substance for *Streptococcus lactis* R and *Lactobacillus casei* has turned out to be a group of compounds. For example, Hutchings *et al.* (23) speak of three "*Lactobacillus casei* factors or 'folic acids' " and Williams (10) mentions "the different folic acids." Mitchell and Williams (37) state that very minor changes in structure of "folic acid" may produce great changes in activity for various organisms. From our work on the isolation of vitamins B<sub>10</sub> and B<sub>11</sub>, both of the vitamins, although distinct entities, seem to be related chemically to the various factors with vitamin B<sub>c</sub> activity, since the properties are so similar and because compounds with vitamin B<sub>c</sub> activity have some vitamin B<sub>10</sub> and B<sub>11</sub> activity when fed alone to the chick.

#### SUMMARY

Methods for the purification of vitamins B<sub>10</sub> and B<sub>11</sub> based on dialysis procedures, chromatogram separation, and Ba(OH)<sub>2</sub>-ethanol precipitations are described. Vitamin B<sub>11</sub> has been concentrated 1600-fold from solubilized liver.

In agreement with our earlier work, vitamins B<sub>10</sub> and B<sub>11</sub> are again differentiated from substances with high vitamin B<sub>c</sub> activity, although such substances do have growth, feathering, and hemoglobin activity when fed alone to chicks at relatively high levels. This action is believed to be largely indirect, as has been shown by work with sulfasuxidine or a corn-meal basal ration and the fact that normal chicks may be obtained with 8  $\gamma$  of vitamin B<sub>c</sub> activity, or less, per 100 gm. of ration.

The existence of another unknown factor necessary to maintain normal hemoglobin formation is suggested.

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## LETTERS TO THE EDITORS

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### ENZYMATIC MICROANALYSIS OF PURINE COMPOUNDS

Sirs:

An enzymatic micromethod for the determination of adenosine and its derivatives has recently been described.<sup>1</sup> The method was based on the observation<sup>2</sup> that adenine and hypoxanthine exhibit a marked difference in light absorption at 265 m $\mu$ . By measuring changes in light absorption at this wave-length, adenosine may be determined with adenosine deaminase, adenylic acid with muscle deaminase, and adenylyl pyrophosphate with the latter enzyme plus potato adenylylpyrophosphatase.

The same principle can be applied to the determination of hypoxanthine, xanthine, and uric acid compounds. Hypoxanthine, xanthine, and allantoin have no absorption or only slight absorption at 290 m $\mu$ ; uric acid, on the other hand, has a very marked absorption maximum at this wave-length.<sup>3</sup> Thus, hypoxanthine may be determined by adding purified xanthine oxidase to a solution containing a few micrograms of hypoxanthine and following the increase in absorption at 290 m $\mu$ . After a few minutes lag period, due to the formation of the intermediate xanthine, the absorption rises steeply and proportionally with time until all hypoxanthine has been oxidized. With xanthine as substrate, the same rise in absorption at 290 m $\mu$  occurs, but without a lag period.

1  $\gamma$  of hypoxanthine (or 0.9  $\gamma$  of xanthine) per ml. upon oxidation causes an increase in extinction at 290 m $\mu$  of +0.075. It is possible to determine 0.1  $\gamma$  of hypoxanthine in 0.1 ml. with an accuracy of about 5 per cent. If inosine is the substrate, no change in the absorption at 290 m $\mu$  takes place when xanthine oxidase is added. If, however, a nucleosidase prepared from rat liver<sup>4</sup> is added together with the oxidase, a rapid increase in absorption at 290 m $\mu$  takes place. If inosinic triphosphate is the substrate, phosphatase must be added in addition to xanthine oxidase and nucleosidase before a rise in absorption occurs.

The method has also been used for studying the liver nucleosidase. It has been found that this enzyme is active only in the presence of inorganic

<sup>1</sup> Kalckar, H. M., *Science*, **99**, 131 (1944).

<sup>2</sup> Holiday, E. R., *Biochem. J.*, **24**, 619 (1930). Myrbäck, K., Euler, H., and Hellström, H., *Z. physiol. Chem.*, **245**, 65 (1932).

<sup>3</sup> Smith, F. C., *Biochem. J.*, **22**, 1499 (1928).

<sup>4</sup> Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707 (1924).

phosphate.<sup>5</sup> The significance and interpretation of this finding will be discussed elsewhere.

By applying the same principles as those used for the hypoxanthine test, it is possible to determine guanine on a micro scale by means of guanase (prepared from rat liver<sup>6</sup>) plus xanthine oxidase at a wave-length of 290 m $\mu$ .

Uric acid may be determined by the decrease in absorption at 290 m $\mu$  which occurs when the compound is oxidized to allantoin by the addition of purified uricase. 1  $\gamma$  of uric acid per ml. upon oxidation causes a decrease in extinction at 290 m $\mu$  of -0.065.

The combination of enzymatic and optical methods for the determination of purine derivatives appears to constitute a rapid, sensitive, and specific technique for the analysis of deproteinized extracts of small (biopsy) samples of normal and pathological tissues. The methods are also useful for the analysis of the purines or nucleosides in nucleic acids, and for the study of enzymes involved in purine metabolism such as deaminases, xanthine oxidase, and the nucleosidases.

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<sup>5</sup> Klein, W., *Z. physiol. Chem.*, **231**, 125 (1935).

<sup>6</sup> Schmidt, G., *Z. physiol. Chem.*, **208**, 185 (1932).

# THE DIFFUSION CONSTANT AND ELECTROPHORETIC MOBILITY OF PHOSPHORYLASES *a* AND *b*\*

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(Received for publication, December 19, 1944)

Phosphorylase *a*, crystallized from dilute salt solutions in the presence of cysteine, exhibits about 65 per cent of its maximum activity in the absence of added adenylic acid (1). Phosphorylase *b* is inactive without added adenylic acid; it may be prepared by allowing the PR (or prosthetic group-removing) enzyme to act on phosphorylase *a* (2).

The molecular weight of crystalline muscle phosphorylase *a* has been determined by Oncley (1) by means of ultracentrifugal and diffusion measurements. In the present paper the two forms of the enzyme are compared with respect to their diffusion constants and migration velocities.

*Diffusion of Phosphorylase b*—The diffusion constant of phosphorylase *b* was determined in the Tiselius apparatus in the manner described by Longworth (3) for crystalline egg albumin. The Tiselius cell with the single long central chamber was filled with protein, the boundaries were shoved out by means of the compensator sufficiently far to be readily visible even after prolonged standing, and the rate of diffusion measured on photographs taken by the schlieren scanning method. Results were calculated by means of the equation  $D = (A^2)/(4\pi t H^2)$  where  $A$  is the area under the curve,  $H$  the maximum height,  $t$  the time in seconds, and  $D$  the diffusion constant in sq. cm. per second (4).

In order to check the method, the diffusion constants for crystallized human serum albumin and horse  $\gamma$ -pseudoglobulin were determined. The human serum albumin used was four times crystallized from ammonium sulfate and was free from carbohydrate and phosphate. The horse  $\gamma$ -pseudoglobulin was precipitated many times with ammonium sulfate and dialyzed repeatedly and the pH adjusted to remove all of the euglobulin; it was finally precipitable in 1.15 M ammonium sulfate at pH 6.6 in the cold, was phosphate-free, contained 1.1 per cent carbohydrate, and showed a single boundary on electrophoresis.

The phosphorylase *b* used in these determinations was prepared by the action of PR enzyme on recrystallized muscle phosphorylase *a*. The PR enzyme was purified to such an extent that an amount of protein equal to 3 per cent of the phosphorylase protein was sufficient to convert the

\* This work was supported by a grant from the Rockefeller Foundation.

phosphorylase *a* to phosphorylase *b*. After the PR action was complete, the enzyme solution was dialyzed at 4° against phosphate buffer at pH 7.1 and 1 per cent KCl. The protein concentration was 4 mg. per cc.

The results are presented in Table I. They are the average of a series of measurements on each of the two boundaries. In the case of phosphorylase *b* the averages of the measurements on each of the two boundaries are recorded separately, because they did not agree as well as in the cases of the other proteins. Seven measurements were made at intervals from 6 to 53 hours. The standard deviation is given in Table I. The values for serum albumin and  $\gamma$ -pseudoglobulin, corrected to 20° on the basis of the viscosity of the solvent, agree satisfactorily with Oncley's determi-

TABLE I  
*Diffusion Constants of Phosphorylase b and Other Proteins*

Protein	Buffer	Ionic strength	pH	Protein	$D_{1,5} \times 10^7$	$D_{2,10} \times 10^7$	$D_{2,20} \times 10^7$
		$\Gamma/2$		mg. per cc.	sq. cm. per sec.	sq. cm. per sec.	sq. cm. per sec.
Serum albumin, crystalline, human	Potassium phosphate	0.1	7.1	9	3.62	5.9	6.1
$\gamma$ -Pseudoglobulin, horse	" "	0.1	7.1	12.5	2.48	4.0	4.1
Phosphorylase <i>b</i> , rabbit muscle	" " + KCl	0.23	7.1	4	$2.0 \pm 0.10$ $2.15 \pm 0.11$	3.2 3.5	
Phosphorylase <i>a</i> , crystalline, rabbit muscle	Na glycerophosphate + KCl + cysteine	0.2	7.0	5			3.2 3.3 3.5

\* Results in the last column are those determined by Oncley (1, 5).

nations (5) on the same proteins. The results on phosphorylase *b* also agree with Oncley's determinations on phosphorylase *a*. The interference due to cystine which was a factor in Oncley's measurements was eliminated and that due to the added PR enzyme was inappreciable. Thus it appears that the diffusion constant, and presumably the molecular weight of phosphorylase *b*, is the same, within the limits of experimental error, as that of phosphorylase *a*.

*Migration Velocity of Phosphorylase*—The crude water extract of rabbit muscle has previously been reported to contain about 2 per cent of phosphorylase. The electrophoretic pattern of water extract is given in Fig. 1, 1 and 2. The small peaks identified by an arrow have the same mobility as reported below for phosphorylase *a*. Electrophoretic pictures (Fig. 1, 3 and 4) are also presented for the 41 per cent ammonium sulfate precipitate.

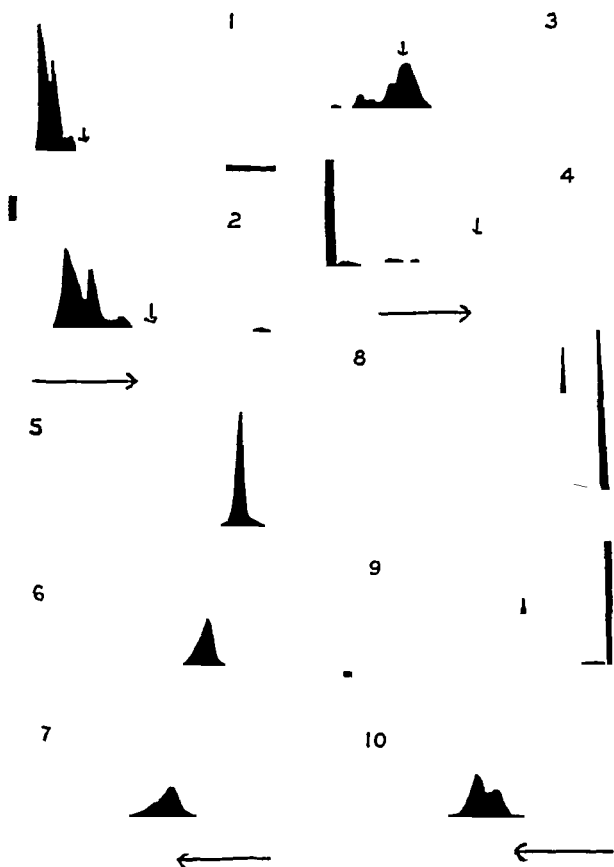


FIG. 1. Photographs of electrophoretic patterns (ascending boundary) of rabbit muscle proteins in phosphate buffers of ionic strength 0.1. 1, 2, original water extract at pH 7.4 and 7.1 volts per cm., migration for 2 and 4 hours respectively. 3, 4, 41 per cent saturated ammonium sulfate precipitate at pH 7.4 and 7.1 volts per cm., migration for 2 and 4 hours respectively. The arrows in 1 to 4 identify phosphorylase *a*. 5, 6, 7, phosphorylase *b* containing some phosphorylase *a* and some impurity at pH 6.56 and 3.5 volts per cm. at 2, 6, and 10 hours respectively. The larger and slower moving peak is phosphorylase *b*. 8, 9, 10, phosphorylase *a* dissolved in phosphorylase *b* at pH 7.1 and 3.5 volts per cm. at 2, 5, and 8 hours respectively. The larger and faster moving peak is phosphorylase *a*. The slower and smaller peak is phosphorylase *b*.

Enzyme activity tests have shown that one-quarter to one-third of this fraction is phosphorylase (1).





# THE ENZYMATIC CONVERSION OF PHOSPHORYLASE *a* TO *b*\*

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The distinctive properties of phosphorylases *a* and *b*, as described in a previous paper (1) and in those of the present series, are summarized in Table I. Phosphorylase *a* is converted to phosphorylase *b* by an enzyme (referred to as PR) which is present in an aqueous extract of muscle and which can be separated from phosphorylase by isoelectric precipitation of PR at pH 5.8. Trypsin at pH 6.0 to 6.2 also converts phosphorylase *a* to *b*.

The present paper deals with the purification and properties of the PR enzyme as well as with the action of PR and trypsin on phosphorylase *a*.

## EXPERIMENTAL

*Purification*—The isoelectric precipitate obtained in the routine preparation of phosphorylase *a* is centrifuged and washed twice with water in the cold room. Most of the protein dissolves when the pH is brought to 8.5 by addition of weak KOH and the precipitate is rubbed vigorously with a glass rod. The solution is frozen solid, and upon thawing a large precipitate is formed which can be discarded without much loss of activity. Fractionation with ammonium sulfate effects considerable purification. An example is given in Table II.

Most of the enzyme is precipitated at 55 to 60 per cent saturation with ammonium sulfate. The dissolved and dialyzed precipitate when frozen yields a large precipitate on thawing which can be discarded without loss of activity. Some inert material still remains which is precipitated by low concentration of ammonium sulfate and is rendered insoluble by freezing and thawing, as shown in step (3) in Table II. The precipitate, obtained between 30 and 56 per cent saturation with ammonium sulfate, when dissolved, dialyzed, and frozen, remains clear after thawing. In some cases the activity per mg. of protein has been raised to 1400 units by ammonium sulfate fractionation.

A dialyzed preparation of a purity level of 1060 units per mg. of protein was brought successively to pH 6.15, 5.7, and 5.2 by the addition of dilute HCl in the cold. The pH was measured on the glass electrode. The precipitate formed at each pH was centrifuged off and analyzed for protein and enzyme activity. The results are shown in Table III. The

\* This work was supported by a grant from the Rockefeller Foundation.

largest amount of enzyme was precipitated between pH 5.7 and 5.2, but no purification was achieved because part of the enzyme was inactivated

TABLE I  
*Comparison of Properties of Phosphorylases a and b*

	Phosphorylase <i>a</i>	Phosphorylase <i>b</i>
Molecular weight calculated from diffusion and ultracentrifugation	$4 \times 10^5$	
Diffusion constant, $D_{20,w} \times 10^7$	3.3	3.3
Electrophoretic mobility, sq. cm. per volt per sec. $\times 10^5$ , phosphate buffer pH 7.15, $\mu$ 0.1; temperature 2°	-3.25	-2.75
Isoelectric point	5.5-5.6	5.8
Solubility at pH 7.2, water	Insoluble	Soluble
0.1 M KCl, 24°	Poorly soluble	"
0.08 M KCl + 0.02 M cysteine, 24°	Soluble	"
0.08 M KCl + 0.02 M cysteine, 0°	Crystallizes	"
Crystal form	Long needles	Rhomboid plates
Activity, without adenylic acid, %	65	None
With adenylic acid, %	100	80
Dissociation constant for combination with adenylic acid, pH 6.7, 25°	$1.5 \times 10^{-8}$	$5 \times 10^{-8}$
Phosphorus content, %	0.08	0.02

TABLE II  
*Sample Protocol of Purification of PR Enzyme*

The isoelectric precipitate obtained at pH 5.8 from the dialyzed extract of 400 gm. of rabbit muscle was dissolved and frozen.

	Protein	Enzyme activity	Units per mg. protein
	mg.	units	
1. After thawing solution, and removing large ppt., 38 cc.	226	62,000	274
2. Solution (1) pptd. with 55% saturated $(\text{NH}_4)_2\text{SO}_4$ ; ppt. dissolved, dialyzed against $\text{H}_2\text{O}$ , and frozen; centrifuged after thawing; ppt. discarded, 17.5 cc.	104	58,500	563
3. Solution (2) pptd. with 30% saturated $(\text{NH}_4)_2\text{SO}_4$ ; ppt. dissolved, dialyzed, and frozen; centrifuged after thawing; ppt. discarded, 7.6 cc.	21	17,200	820
4. Supernatant fluid of 30% saturated $(\text{NH}_4)_2\text{SO}_4$ brought to 56% saturation; ppt. dissolved, dialyzed, and frozen; clear after thawing, 7 cc.	29	26,500	910

at the acid pH. This is shown by the fact that for a recovery of 90.7 per cent of the protein there was regained only 66.1 per cent of the enzyme activity.

Stability tests also indicated a loss of activity at acid pH in enzyme solutions of about the same purity level as those used in the experiment in Table III. When kept at 28.5° for 2.5 hours, the enzyme lost about 30 per cent of its activity at pH 8.4 to 7.1 and 80 per cent at pH 5.9. At pH 7.1 and 5° the enzyme lost 47 per cent of its activity in 5 days. Heating at pH 7.1 to 56° for 10 minutes caused a loss of 42 per cent of PR activity. The pH optimum for activity was between 6.8 and 7.0; considerable enzyme activity could be demonstrated at pH 6 and 7.4.

*Pentose Content of PR Enzyme Fractions*—It was found that the isoelectric precipitate (pH 5.8) of a dialyzed muscle extract contained ribonucleic acid. Crude PR preparations showed 6 to 7  $\gamma$  of pentose per mg. of protein, when the determinations were carried out by the method of

TABLE III  
*Effect of Isoelectric Precipitation*

A dialyzed PR preparation was used which contained 5 mg. of protein and 5300 units per cc., corresponding to 1060 units per mg. of protein. For the description see the text.

pH at which precipitate was collected	Protein recovered	Enzyme units recovered	Units per mg. protein
	<i>per cent</i>	<i>per cent</i>	
6.15	14.7	13.7	980
5.7	20.8	15.7	800
5.2	19.7	21.3	1140
Supernatant fluid of pH 5.2 ppt.	35.5	15.4	470
	90.7	66.1	

Mejbaum (2) with muscle adenylic acid as standard.<sup>1</sup> After purification the pentose content was reduced to 2 to 3  $\gamma$  per mg. of protein. In a fractionation the precipitates formed with 26 to 38, 38 to 50, and 50 to 62 per cent saturated ammonium sulfate contained 1.8, 2.9, and 5.8  $\gamma$  of pentose per mg. of protein, respectively. The most active enzyme was in the middle fraction. After incubation with the crystalline ribonuclease of Kunitz (3), 80 to 100 per cent of the pentose appeared in the trichloroacetic acid filtrate of the proteins, while before incubation with ribonuclease all of the pentose remained in the trichloroacetic acid precipitate. The PR activity was not affected by incubation with ribonuclease, which split most of the nucleic acid.

*Kinetics of PR*—Since the original observations were made it has been

<sup>1</sup> On the basis of phosphorus content, yeast nucleic acid gives only 40 per cent of the pentose color of muscle adenylic acid.

found that the enzyme has very low activity in the absence of cysteine and that its activity in the presence of cysteine is accelerated 2 to 3 times by manganese ions. It was also found that 0.03 M glycerophosphate, the buffer which was used in previous experiments, markedly inhibits PR activity and changes the kinetics of the enzyme.

Under specified conditions the conversion of phosphorylase *a* to *b* by the PR enzyme follows the first order reaction rate equation; this permits the PR enzyme units to be expressed in terms of the velocity constant.

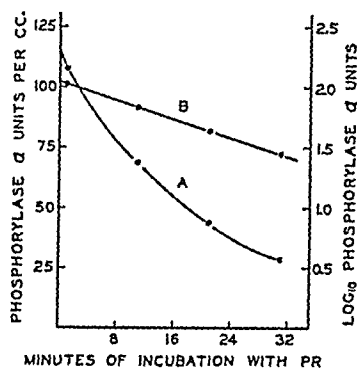


FIG. 1

FIG. 1. Effect of PR enzyme during incubation with phosphorylase *a*. 1 cc. of reaction mixture contained 80  $\gamma$  of phosphorylase *a* and 16  $\gamma$  of PR protein in 0.03 M cysteine at pH 6.8. Incubated at 26°. Curve A, time of incubation plotted against phosphorylase *a* units. Curve B, time of incubation plotted against  $\log_{10}$  of phosphorylase *a* units. For a description see the text.

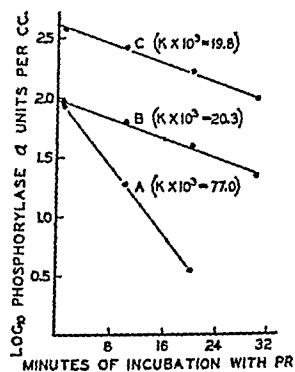


FIG. 2

FIG. 2. The  $\log_{10}$  of the concentration of phosphorylase *a* was plotted against time during incubation with PR enzyme at pH 6.8 and 26°. Curve A, 4 times as much PR and the same initial phosphorylase *a* concentration as for Curve B. Curve C, the same amount of PR and 4 times as much phosphorylase *a* as for Curve B.  $K \times 10^3$  calculated from the slope. In the experiment of Curve C 190  $\gamma$  of phosphorylase *a* and 13.6  $\gamma$  of PR protein were present per cc. of reaction mixture.

An example is given in Fig. 1. Aliquots of the reaction mixture were removed at stated intervals and tested for their phosphorylase *a* activity (in the absence of adenylic acid). In the phosphorylase *a* test (4) the glucose-1-phosphate concentration is 0.016 M, the glycogen concentration 1 per cent, and the incubation period 5 minutes. Since no adenylic acid is added, phosphorylase *b* (which is formed by the action of the PR enzyme) does not act. Divalent ions (among others glucose-1-phosphate) inhibit the PR enzyme and their concentration is sufficiently high in the phosphorylase *a* test to prevent further action of PR on phosphorylase *a*. When the  $\log_{10}$  of the phosphorylase *a* concentration is plotted against time, a straight

line is obtained.  $K \times 10^3$  for the experiment in Fig. 1 is equivalent to 19.4 PR units per cc. or to 1210 units per mg. of protein.<sup>2</sup>

In Fig. 2, Curves B and C, is shown an experiment in which the initial phosphorylase *a* concentration was varied 4-fold. The per cent of phosphorylase *a* converted to *b* was within these limits independent of the initial phosphorylase *a* concentration, as shown by the values of  $K$ . In Curve C, the PR protein fraction added (undoubtedly quite impure) was only 6.7 per cent of the total protein in the reaction mixture; nevertheless, 76 per cent of phosphorylase *a* was converted to *b* in 30 minutes at 26°. It follows that a relatively small contamination with PR can lead to serious losses in the preparation of phosphorylase *a* crystals.

In Curve A, Fig. 2, it is shown that the reaction remains first order even when the phosphorylase *a* concentration has fallen to 5 per cent of its original value and that the value of  $K$  is proportional to the dilution of the PR enzyme.

In previous experiments (1) the PR activity appeared relatively weak because it was measured in a 0.03 M glycerophosphate buffer. The experiment in Fig. 3 shows that the addition of 0.03 M phosphate esters to the reaction mixture inhibits the PR activity. After 10 minutes of incubation with PR alone about 80 per cent of phosphorylase *a* had disappeared, in the presence of phosphate esters only 10 to 20 per cent. The order of inhibition was mannose-1-phosphate > glycerophosphate > glucose-1-phosphate.<sup>3</sup> The inhibition is not specific for phosphates since ammonium sulfate (0.03 M) has also been found to inhibit the PR activity.<sup>4</sup>

<sup>2</sup> Protein was determined by the method of Robinson and Hogden (5).

<sup>3</sup> When glucose-1-phosphate was the inhibiting agent, only glycogen was added in the phosphorylase test to start the reaction. It was found that the order of addition of the components of the phosphorylase system makes an appreciable difference in the initial reaction rate. When phosphorylase or glucose-1-phosphate was added as the last component to the reaction mixture, the initial rate of the reaction, measured at 1, 3, and 5 minutes, was the same and followed the first order reaction rate. When glycogen was added as the last component, the rate of the reaction was initially much slower than in the other two cases and increased with time, giving a slightly S-shaped rate curve.

<sup>4</sup> These results have some bearing on the preparation of phosphorylase *a* crystals. As described previously, the PR enzyme is separated from phosphorylase by precipitation at pH 5.8. Any PR which is not removed in this manner is concentrated along with phosphorylase by precipitation with ammonium sulfate. When the ammonium sulfate precipitate is dissolved and dialyzed, the PR will begin to act only when the salt concentration has dropped considerably. At that point crystallization of phosphorylase *a* removes it from the action of PR. It should be emphasized in this connection that, although cysteine markedly accelerates the activity of PR, it is necessary for the preparation of phosphorylase *a* in crystalline form. Once the enzyme has been crystallized, it can be recrystallized once or twice without adding cysteine.

That one is dealing with a salt effect is shown by the fact that glucose, in contrast to glucose-1-phosphate, does not inhibit the PR activity. Glycogen is also without effect.

Fig. 3 also shows that when PR is acting in the presence of inhibitory salts the reaction is no longer first order, but is linear with time.

*Effect of Cysteine, Manganese, and Other Ions*—In order to test the effect of cysteine on PR activity, phosphorylase *a* crystals (which are kept in the cold suspended in a cysteine-glycerophosphate buffer) were washed repeatedly with 0.03 M KCl in the cold room. The enzyme was dissolved in bicarbonate solution at 35° and an insoluble residue consisting mostly of cystine was discarded. Manganese ions and PR enzyme were added, and an aliquot of this mixture was made 0.015 M with respect to cysteine. The pH in both samples was 7.1. The sample incubated without cysteine showed only a trace of PR activity during 50 minutes of incubation at 26°.

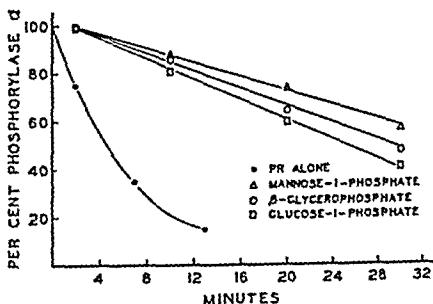


FIG. 3. Effect of incubation of phosphorylase *a* with PR (45.5  $\gamma$  per cc.) in the presence and absence of 0.03 M phosphate esters, pH 6.7 and 27°.

In the sample incubated with cysteine the PR activity was rapid and led to an almost complete disappearance of phosphorylase *a* during 20 minutes of incubation. The activity of the PR enzyme was accelerated 15 to 20 times by the presence of cysteine.

The optimal concentration of  $Mn^{++}$  ions was from 2.5 to  $5 \times 10^{-4}$  M and caused a 2- to 3-fold increase in PR activity.  $Mg^{++}$  ions, in these concentrations, were inhibitory, while  $Co^{++}$  ions had no effect. For example, for a particular PR preparation with no ions added  $K \times 10^3$  was 31, while with  $5 \times 10^{-4}$  M of the following ions it was  $Mn^{++}$  74,  $Mg^{++}$  20,  $Co^{++}$  30. In the presence of 0.03 M glycerophosphate  $Mn^{++}$  ions did not have an accelerating effect on PR activity.

*Effect of Trypsin*—Trypsin converts phosphorylase *a* to *b* at a rate which falls off more rapidly than predicted by a first order reaction and the activity is not directly proportional to the trypsin concentration. Most experiments were performed at pH 6.2, because at that pH the proteolytic

activity of trypsin is reduced to a minimum. A few experiments were carried out at lower (6.0) and higher (6.5) pH without any marked change in the kinetics of the reaction.  $Mn^{++}$  ions did not accelerate the trypsin action on phosphorylase *a*.

On comparing the relative activities of crystalline trypsin and of PR (per mg. of protein), one finds that the best preparations of the latter (in the presence of  $Mn^{++}$  ions) are about one-half as active as trypsin, if the initial rate of the reaction is taken as a measure. If the time for complete conversion of phosphorylase *a* to *b* is taken as a basis of comparison, PR is about as active as trypsin.

As in the case of the experiments with the PR enzyme, the action of trypsin on phosphorylase *a* did not continue during the phosphorylase test. An example is given in Table IV. In Experiment 1 a concentration of 4  $\gamma$  of trypsin per cc. caused a rapid disappearance of phosphorylase *a*, about 75 per cent during 20 minutes of incubation. The time curve for the action of trypsin would not be valid if trypsin continued to act during the phosphorylase test, which lasts 5 minutes. That this error did not arise is shown in Table IV, Experiment 2. A time curve for phosphorylase activity in the presence of the same concentration of trypsin did not show an appreciable falling off in rate in 50 minutes. The inhibitory effect of glucose-1-phosphate on the action of trypsin on phosphorylase *a* is probably responsible for the lack of action of trypsin under these conditions. An example is given in Fig. 4; it may be seen that glucose-1-phosphate is much more inhibitory than either mannose-1-phosphate or glycerophosphate.

*Enzymatic Tests for Adenylic Acid*—In a previous paper (1) an attempt was reported to demonstrate free adenylic acid in a digestion mixture of phosphorylase *a* and trypsin. Although the results were negative, it was decided to repeat the experiments with a somewhat modified technique. PR enzyme preparations could not be used for this purpose because they contained adenylic deaminase.

A digestion mixture of phosphorylase *a* and trypsin was ultrafiltered with air pressure through a cellophane membrane (a) without and (b) with the addition of a known amount of adenylic acid. The ultrafiltrates were tested for the presence of free adenylic acid by adding them to the phosphorylase *a* test system which had been standardized with known amounts of adenylic acid. The test system showed 34.2 per cent splitting of 1-ester in 5 minutes without, 46.6 per cent with  $3 \times 10^{-6}$  M, and 55 per cent with  $10^{-5}$  M adenylic acid. Ultrafiltrate (a), when added to the test system, gave a splitting of 34.2 per cent, ultrafiltrate (b) of 52 per cent. By calculation the former should have given a final concentration of adenylic acid of  $6 \times 10^{-6}$  M (assuming 1 adenylic acid molecule per molecule of enzyme) and the latter (assuming that no adenylic acid was



set free by trypsin) of  $3 \times 10^{-6}$  M. The experiment shows that the added adenylic acid was recovered in the ultrafiltrate. There was no evidence for the liberation of adenylic acid when phosphorylase *a* was incubated with trypsin.

TABLE IV  
*Effect of Trypsin on Phosphorylase a*

In Experiment 1 phosphorylase *a* was incubated with 4  $\gamma$  of crystalline trypsin per cc. in 0.03 M cysteine, pH 6.2 at 27°. Samples were withdrawn at stated intervals in order to determine the phosphorylase *a* concentration. In Experiment 2 phosphorylase *a* was acting on glucose-1-phosphate at pH 6.2 and 27° without and with the addition of 4  $\gamma$  of trypsin per cc.

Experiment 1		Experiment 2					
Phosphorylase incubated with trypsin		Phosphorylase alone			Phosphorylase + trypsin		
Time of incubation	Phosphorylase <i>a</i> concentration	Time	1-Ester converted	$K \times 10^3$	Time	1-Ester converted	$K + 10^3$
min.	per cent	min.	per cent		min.	per cent	
0.5	94	5	8.5	9.3	5	8.3	9.0
10	38	10	16.1	9.2	10	15.4	8.8
20	25	20	29.5	9.4	20	28.0	8.8
30	18	32	41.6	9.3	32	40.2	8.8
		50	55.0	9.2	50	51.7	8.3

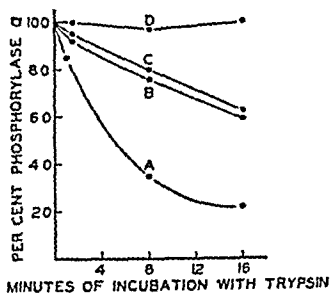


FIG. 4. Effect of incubation of phosphorylase *a* with crystalline trypsin (12.5  $\gamma$  per cc.) in the presence and absence of 0.03 M phosphate esters, pH 6.2 and 28°. Curve A, trypsin alone; Curve B, glycerophosphate; Curve C, mannose-1-phosphate; Curve D, glucose-1-phosphate.

*Liberation of Organic Phosphate from Phosphorylase a*—Previous analyses (1) showed that phosphorylase *a* contained an average of 0.7  $\gamma$  and phosphorylase *b* an average of 0.2  $\gamma$  of organic P per mg. of protein. If this difference were significant, one would expect that PR would liberate

phosphate while acting on phosphorylase *a*. This has been found to be the case.

The molybdate-stannous chloride method described by Fontaine (6) was used; it permits the determination of 0.5 to 1  $\gamma$  of P and has the advantage that the sulfuric acid concentration is high (final concentration 2 *N*), which is favorable in the ashing of protein. Colorimetric readings were made at wave-length 820  $m\mu$  in the Beckman spectrophotometer or at 660 in the Klett-Summerson photoelectric colorimeter; the agreement of the P calculated from these two readings was satisfactory.

Five to six times recrystallized phosphorylase *a* was used for these experiments. In some cases the usual glycerophosphate-cysteine buffer was replaced by a succinate-cysteine buffer for recrystallizations; in other cases the whole preparation was carried through with the latter buffer; finally the crystals were washed in the centrifuge in the cold with repeated changes of 0.03 *M* KCl. If glycerophosphate was used for the last crystallization, the washings were continued until calculations showed that the glycerophosphate would have been diluted beyond the limit of detection. There was no essential difference in the total P content of these different preparations (see Table V). When phosphorylase *a* was incubated with the intestinal phosphatase preparation of Schmidt and Thannhauser (7), about 5 per cent of the total organic P appeared as inorganic P; a conversion of phosphorylase *a* to *b* did not occur.

Various methods of deproteinization were tried, but they all yielded organic P in the protein-free filtrate. When precipitation with trichloroacetic acid (final concentration 2.5 per cent) was carried out in an ice bath and the protein centrifuged off in the cold room, one-half as much P was found as when the operations were carried out at room temperature (Table V).

Precipitation with trichloroacetic acid in the cold was used in the experiments with the PR enzyme and with trypsin, except in one case in which the P split off was removed by dialysis and was determined in the dialysate. Table VI shows that most of the phosphate appears in the protein-free filtrate when phosphorylase *a* is converted to form *b* by the PR enzyme or by trypsin and that there exists a parallelism between the per cent conversion of phosphorylase *a* to *b* and the liberation of phosphate. The organic P compound is dialyzable and is difficult to hydrolyze in acid (zero hydrolysis in 10 minutes in *N*  $H_2SO_4$  at 100° and about 75 per cent in 4 hours). Further identification was precluded by the small amount of material available. No characteristic absorption spectrum in the ultra-violet was detected for the material split off by PR.

PR preparations do not show any proteolytic activity in Anson's test (8) with hemoglobin as substrate. No measurable amount of non-protein

nitrogen is liberated from dialyzed rabbit serum, muscle protein fractions, or phosphorylase *a* during incubation with PR. The determinations were made on trichloroacetic acid filtrates after ashing, distillation of ammonia,

TABLE V

*Phosphorus Content of Phosphorylase a and of Filtrates Obtained after Precipitation of Protein*

P was determined after ashing of protein or filtrate and is expressed in micrograms per mg. of protein. For the description see the text.

Phosphorylase <i>a</i> preparation No.	Total P	P in filtrate (CCl <sub>3</sub> COOH)		P in other filtrates
		0°	25°	
83	0.73	0.29	0.59	
84	0.87	0.25	0.45	
85	0.93	0.22	0.57	
86	0.72	0.25	0.54	0.31 (HgCl <sub>2</sub> )
87	0.75			0.33 "
96	0.85	0.26	0.52	0.29 (Heat)
	0.80	0.25	0.53	

TABLE VI

*Total P in Filtrates and in Protein Precipitate after Incubation of Phosphorylase a with PR Enzyme or with Trypsin*

Filtrates were prepared by precipitation of protein with CCl<sub>3</sub>COOH in the cold.

Phosphorylase <i>a</i> preparation No.	Time of incubation with PR or trypsin (Tr)	Phosphorylase <i>a</i> converted to <i>b</i>	P in CCl <sub>3</sub> COOH filtrate	P in CCl <sub>3</sub> COOH precipitate
	min.	per cent	γ per mg. protein	γ per mg. protein
84	45 (PR)	70	0.57	0.31
85	30 (Tr)	90	0.61	
	60 "	100	0.68	0.25
86	60 (PR)	90	0.45*	0.30*
87	60 "	85	0.56	
	120 "	100	0.69	0.20
96	20 "	75	0.51	
	45 "	95	0.65	
	120 "	100	0.67	0.21
Average (for 100 % conversion)			0.68	0.22

\*No protein precipitant was used. P was determined in the dialysate and in the fluid remaining in the dialyzing bag, respectively.

and nesslerization. Peptidase activity could not be detected when PR was incubated with leucylglycine or triglycine in the presence of Mn<sup>++</sup> ions and with and without the addition of cysteine.

## DISCUSSION

The nature of the prosthetic group which is split off by PR or trypsin from phosphorylase *a* has not been elucidated. All attempts to separate free adenylic acid (or pentose) among the split-products gave negative results. An average value of 0.3  $\gamma$  of pentose per mg. of protein has been reported (1) for four times recrystallized material; this value has been reduced further by additional recrystallizations. While 0.7  $\gamma$  of P per mg. of phosphorylase *a* is split off by PR or trypsin, 0.3  $\gamma$  of pentose would be equivalent to only 0.06  $\gamma$  of P. The question whether or not phosphorylase *a* contains adenylic acid must therefore be left open, in spite of the fact that the activity of phosphorylase *b* can be restored by addition of adenylic acid.

That the principle involved in the action of the PR enzyme, namely, the splitting off of a prosthetic group from another enzyme, may be of physiological importance is shown in the paper which follows. It seems probable that other PR enzymes exist. Ratner, Nocito, and Green have found recently that the flavoproteins, glycine oxidase and *D*-amino acid oxidase, may be prepared from kidney in two forms, one of which is active without addition of flavin adenine dinucleotide, while the other requires the addition of the nucleotide for its activity. The two forms are comparable to phosphorylases *a* and *b*, but as yet no enzyme has been isolated from tissues which effects the transformation of one oxidase into the other *in vitro*.

## SUMMARY

1. The PR enzyme which catalyzes the reaction, phosphorylase *a*  $\rightarrow$  phosphorylase *b*, has been purified by fractionation with ammonium sulfate.
2. The PR enzyme has very low activity in the absence of phosphate. Its activity in the presence of cysteine is accelerated 2 to 3 times.  $Mn^{++}$  ions.  $Mg^{++}$  ions in this concentration are slightly activating, while  $Co^{++}$  ions have no effect. Various phosphate esters (phosphate, glucose-1-, and mannose-1-phosphate) as well as other anions such as sulfate in 0.03 M concentration inhibit the PR enzyme.
3. The conversion of phosphorylase *a* to phosphorylase *b* by crystallization follows the first order reaction rate equation over a wide range of phosphorylase *a* concentrations. This permits the rate to be expressed in terms of the first order velocity constant.
4. The conversion of phosphorylase *a* to *b* by crystallization at pH 6.2 is not a first order reaction and is not accelerated by phosphate esters (0.03 M) inhibit the trypsin effect.
5. The PR enzyme and trypsin split off from phosphorylase *a* a prosthetic group which contains organic phosphate. The amount of

split off is parallel to the extent of conversion of phosphorylase *a* to *b* by these agents, and when complete amounts to 0.7  $\gamma$  per mg. of protein. The phosphate compound is dialyzable and difficult to hydrolyze in acid.

The authors are indebted to Dr. Schmidt for a preparation of intestinal phosphatase, to Dr. Kunitz for crystalline ribonuclease, and to Dr. Fruton for leucylglycine and triglycine.

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# THE EFFECT OF STIMULATION AND RECOVERY ON THE PHOSPHORYLASE *a* CONTENT OF MUSCLE\*

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(Received for publication, December 19, 1944)

It has been mentioned before (1) that an extract of resting muscle contains mainly phosphorylase *a*, while stimulated muscle yields mainly phosphorylase *b*. Data bearing on this observation are presented in detail in this paper.

## EXPERIMENTAL

The determination of phosphorylases *a* and *b* in mixtures is based on two parallel activity determinations, one without and one with the addition of adenylic acid ( $10^{-3}$  M). In the former case phosphorylase *a* shows, on an average, 65 per cent of its full activity, while phosphorylase *b* is inactive; in the latter case phosphorylases *a* and *b* show full activity (1). Activity measurements were carried out as described previously, and enzyme units per gm. of muscle were calculated from the first order velocity constant (2).

Results for twelve consecutive preparations of phosphorylase from muscles of resting animals are recorded in Table I. Rabbits were anesthetized by intravenous injection of pentobarbital and the muscles of legs and back removed with as little stimulation as possible. The extraction of muscle with 2 volumes of water and the separation of phosphorylase were carried out as outlined in a previous paper (3).

As soon as the crude extract was obtained, about  $1\frac{1}{2}$  hours after the excision of the muscles, an aliquot was diluted twenty-five to fifty times with 0.03 M cysteine, pH 6.8, for activity measurements. Under these conditions neither phosphoglucomutase, the enzyme which catalyzes the reaction glucose-1-phosphate  $\rightleftharpoons$  glucose-6-phosphate, nor inorganic phosphate which is present in the extract interferes seriously. A small blank reading for inorganic phosphate was deducted from the inorganic phosphate formed during the reaction. Column 3, Table I, indicates that with one exception (Experiment 75) 80 to 100 per cent of the phosphorylase extracted from resting muscle was present in the *a* form, with an average of 92 per cent.

There were traces of adenylic acid present in the crude extract. This

\* This work was supported by a grant from the Rockefeller Foundation.

is shown by the fact that for the eight cases in Column 3, Table I, in which 100 per cent phosphorylase *a* was calculated to be present, the average ratio (activity without adenylic acid)/(activity with adenylic acid)  $\times 100$  was 80 instead of 65. A concentration of  $1.5 \times 10^{-6}$  M adenylic acid gives an activity ratio of about 78 for phosphorylase *a* (2). Such

TABLE I

*Phosphorylases a and b in Extracts of Resting Rabbit Muscle*

Activity measurements were made at 30° in the presence and absence of adenylic acid with extracts diluted 25 to 50 times with 0.03 M cysteine, pH 6.8. For the description see the text.

Experiment No. (1)	Crude extract		Dialyzed extract*		Dialyzed ammonium sulfate ppt.	
	Phosphorylase <i>a</i> + <i>b</i> per g muscle† (2)	(3)	(4)	(5)	(6)	(7)
	units	per cent	units	per cent	units	per cent
69	1290	100	1420	100	1130	79
70	1710	83	1330	74		
71	1500	100	1450	100	1400	83
72	690	91	620	100	470	87
73	1020	100	990	100	930	88
74	2110	100	1620	100	1540	98
75	830	45	870	68	860	54
76	1020	80	840	81	840	52
77	1010	100	1150	100	1010	95
78	1290	100	1260	100	1210	73
79	1860	100	1770	100	1960	95
80	1740	100	1810	100	1800	100
	1340	92	1260	93	1195	82

\* The precipitate which forms on adjusting the dialyzed extract to pH 5.8 was removed.

† The precipitate formed at 41 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$  was dialyzed against cysteine-glycerophosphate buffer and an aliquot of phosphorylase *a* crystals suspended in the mother liquor was taken for activity measurement.

‡ Calculated from activity measurements in the presence of adenylic acid.

§ Calculated from the ratio (activity without adenylic acid)/(activity with adenylic acid)  $\times 100$ . A ratio of 65 represents 100 per cent phosphorylase *a*.

low concentrations of adenylic acid are without effect on the activity of phosphorylase *b*.

After dialysis for 3 to 4 hours at low temperature (5°) and removal of the precipitate obtained at pH 5.8 there was no appreciable loss of total phosphorylase (Column 4, Table I) or of the percentage of phosphorylase

*a* (Column 5). The activity ratio dropped to 71, indicating removal of adenylic acid during dialysis.

Precipitation of phosphorylase with 41 per cent saturated ammonium sulfate is complete, since an average of 95 per cent of the activity present in the dialyzed extract is recovered in the dialyzed ammonium sulfate precipitate. The Tiselius pictures in Fig. 1 (1 to 4) in the accompanying paper by Green show how great a purification of phosphorylase is accomplished by this one step.

PR enzyme that has escaped precipitation at pH 5.8 is included in the ammonium sulfate precipitate and is concentrated along with phosphorylase. During the dialysis of the ammonium sulfate precipitate some PR action takes place before phosphorylase *a* crystallizes out. PR is without effect on the crystals of phosphorylase *a*. It is to be noted that the activity measurements were made by stirring up the crystals in the mother liquor and taking an aliquot for analysis. Column 7 of Table I shows that the average of phosphorylase *a* has fallen from 93 to 82 per cent owing to the action of the PR enzyme.

The preparation of phosphorylase is unique among tissue enzyme preparations, since under optimal conditions 60 to 70 per cent of the enzyme present in the crude extract of resting muscle can be recovered as phosphorylase *a* crystals.

In the majority of cases phosphorylase *a* crystals cannot be obtained from previously stimulated muscle. This is shown in Table II. In the first series of experiments a lethal dose of strychnine sulfate was injected intravenously. The animals were sacrificed after the first convulsive seizure, which lasted from 1 to 2 minutes. Back and leg muscles were analyzed separately, because it was noted that the former showed a more sustained contraction than the latter.

The crude or dialyzed extract and the dialyzed ammonium sulfate precipitate of the back muscles of strychninized rabbits contained only from 3 to 15 per cent of phosphorylase *a*, the remainder being phosphorylase *b*. When such a large amount of phosphorylase *b* is present, it holds phosphorylase *a* in solution, and the latter does not crystallize out.<sup>1</sup> Table II, last column, shows that unless at least 25 per cent of the total phosphorylase content is in the *a* form no crystals are obtained. With one exception the leg muscles of the strychninized rabbits, like the back muscles, yielded very low values for phosphorylase *a*.

In the experiments with electrical stimulation the animals were anesthetized with pentobarbital and the leg nerves prepared on one side. A tetanizing current was applied for 2 to 3 minutes, until the muscles showed

<sup>1</sup> This fact was made use of in the preceding paper by Green to determine the electrophoretic mobilities of mixtures of phosphorylases *a* and *b*.



fatigue. It was difficult to stimulate all the leg muscles with equal intensity unless a rather strong current was used, and this led to a considerable spread of stimulation to the resting leg and in some cases to the back muscles. The latter were used as a control and showed an average of 59 per cent of phosphorylase  $\alpha$ , which is considerably less than the

TABLE II

*Effect of Strychnine Convulsions and of Electrical Stimulation on Phosphorylase  $\alpha$  Content of Rabbit Muscle*

The activity measurements were carried out as indicated in Table I. For the description see the text.

Experiment No.	Procedure and type of muscle	Crude extract		Dialyzed extract		Dialyzed ammonium sulfate precipitate		
		Phosphorylase $\alpha + \beta$ per gm. muscle	Phosphorylase $\alpha$	Phosphorylase $\alpha + \beta$ per gm. muscle	Phosphorylase $\alpha$	Phosphorylase $\alpha + \beta$ per gm. muscle	Phosphorylase $\alpha$	Phosphorylase $\alpha$ crystals
		units	per cent	units	per cent	units	per cent	
A	Strychnine, back	2310	6	2100	11			
	“ leg	1720	2	1840	9			
B	“ back	1510	10	1620	5			
	“ leg	830	93	860	69			
C	“ back	930	6	980	0			
	“ leg	1250	11	1100	20			
67	“ back	3180	16			2850	6	—
64	“ back					1620	3	—
	“ leg					1130	25	+
66	“ back					1780	5	—
	“ leg					2130	5	—
61	Resting, back					1780	71	+
	Electrical stimulation, leg					850	20	—
62	Resting, back					1180	29	+
	Electrical stimulation, leg					1740	1	—
63	Resting, back					1290	68	+
	Electrical stimulation, leg					960	2	—
68	Resting, back	1370	100			900	65	+
	“ leg	950	80			660	51	+
	Electrical stimulation, gastrocnemius	930	23			610	17	—

corresponding average of 82 per cent recorded in Table I. The electrically stimulated leg muscles showed an average phosphorylase  $\alpha$  content of 10 per cent and yielded no crystals. In one case, in order to make the time interval between stimulation and measurement of enzyme activity as short as possible, only one stimulated muscle (gastrocnemius) was

excised and extracted (Experiment 68, Table II); analysis of the crude extract showed a low phosphorylase *a* content, when compared with the back muscles and the resting muscle of the opposite leg.

It will be noted in Tables I and II that there is a marked individual variation in the phosphorylase content. The extraction with 2 volumes of water is incomplete and yields about 75 per cent of the phosphorylase present, but since the extraction procedure was strictly standardized, the results are comparable among themselves. On an average the total phosphorylase content of the stimulated muscle in Table II did not differ significantly from that of the resting muscle in Table I.

Experiments similar to those on rabbits were carried out on frogs. In order to obtain resting muscles the spinal cord was transected in the lumbar region. The legs were skinned and the muscles excised, weighed, and extracted in the manner described for rabbit muscles. The undialyzed extracts were generally tested within  $\frac{1}{2}$  to 1 hour after the dissection of muscle. Dialysis was for 3 to 4 hours at 8°. Stimulation of both legs was effected by holding an electrode connected with an inductorium against the skin above the lumbar spine. An interrupter served to deliver 60 stimuli per minute. Fatigue became noticeable after 30 to 60 twitches, depending on the general state of the frogs. At the end of 90 twitches the cord in one group of frogs was transected immediately and the muscles excised, while another group was first allowed to recover for 5 to 60 minutes in a moist environment.

Several batches of frogs were received between May and July, 1943. When freshly shipped they were in good condition and the phosphorylase content per gm. of muscle was about the same as in rabbits. After having been without food for 2 or more weeks, they became thin and the phosphorylase content of the muscles was much lower. Two frogs were chosen each day, one serving for resting muscle and the other for stimulated muscle with or without recovery.

Table III shows that extracts of resting frog muscle contained a high percentage of phosphorylase *a* and that electrical stimulation resulted in a marked decrease in phosphorylase *a* without significant change in the total phosphorylase content. The effect of electrical stimulation was quickly reversible. When the stimulated muscles were allowed to recover in the intact animal for a period of 10 to 15 minutes, the phosphorylase *a* content returned to the resting level. A period of 5 minutes rest was insufficient for complete recovery.

It will be noted that in the four experiments in which the muscles were allowed to recover for 5 minutes the average phosphorylase *a* content in the undialyzed extract was 43 per cent, while the same extracts after 4 hours of dialysis at 8° showed an average of 64 per cent. In the other

experiments in Table III the difference was less marked, although the average percentage of phosphorylase *a* in the dialyzed extracts was in all cases slightly higher than in the undialyzed extracts. An occasional extract of rabbit muscle shows the same behavior (*cf.* Experiment 75, Table I). The reason for the apparent increase in phosphorylase *a* has not been ascertained.

The possibility had to be considered that from stimulated muscle more PR is extracted than from resting muscle. If this were the case, the conversion of phosphorylase *a* to *b* might take place in the extract in the time interval between its preparation and testing, rather than in the living muscle. The PR content of a resting and a stimulated muscle of a rabbit, the former containing mostly phosphorylase *a* and the latter only phosphorylase *b*, was determined in the following manner. To each

TABLE III

*Phosphorylase a and b Content of Muscles of Summer Frogs at Rest, after Electrical Stimulation, and after Stimulation and Recovery*

Stimulation consisted of 90 twitches of both hind legs in the intact animal in a period of 1½ minutes. Enzyme activity measurements were carried out as indicated in Table I. For the description see the text.

No. of frogs	Procedure	Undialyzed extract		Dialyzed extract	
		Phosphorylase <i>a</i> + <i>b</i> per gm. muscle	Phosphorylase <i>a</i>	Phosphorylase <i>a</i> + <i>b</i> per gm. muscle	Phosphorylase <i>a</i>
		units	per cent	units	per cent
14	Resting	1290	86		
7	Stimulated	1310	37		
4	" 5 min. recovery	1330	43	1440	64
2	" 10 and 15 min. recovery	1290	82	1240	91
13	Resting			1350	91
17	Stimulated			1240	40
5	" 20 to 60 min. recovery			1140	94

of the extracts was added crystalline phosphorylase *a* and the mixtures were incubated at 27° for 2½ hours. About one-half of the added phosphorylase *a* was converted to phosphorylase *b* during this period.  $K \times 10^3$  calculated as described in the preceding paper was equivalent to 5.4 PR units per cc. of extract of the stimulated and to 4.8 units per cc. of extract of the resting muscle. The PR activity in the extract of the resting and the stimulated muscle thus did not differ appreciably. The low phosphorylase *a* content which is found in stimulated muscle cannot be explained by PR activity after extraction.

enzyme (PR) can be extracted from muscle which effects this conversion *in vitro* and which may be regarded as responsible for the conversion *in vivo*. Phosphorylase *a* is active in the absence of adenylic acid, while phosphorylase *b* requires a concentration of  $5 \times 10^{-5}$  M adenylic acid for half maximal activity. Data published by Lohmann and Schuster (4) make it doubtful whether rabbit or frog muscle contains such a concentration of free adenylic acid.

As a consequence of the conversion of phosphorylase *a* to *b* the rate of glycogen breakdown during activity would be slowed down, and this may represent a regulatory mechanism which would prevent the exhaustion of glycogen stores in fatigue. Experiments on frogs indicate that the phosphorylase *a* content of muscle is quickly restored during a short period of rest. This supports the idea that a regulatory mechanism is involved.

#### SUMMARY

1. Resting muscle of rabbits contains mainly phosphorylase *a*, as shown by an analysis of crude muscle extract as well as by the preparation of phosphorylase *a* crystals in high yield.

2. During strong muscular contractions produced by strychnine or by electrical stimulation most of the phosphorylase *a* is converted to phosphorylase *b* by the action of the PR enzyme *in vivo*. Crystalline phosphorylase *a* cannot be prepared from extracts of muscles stimulated to fatigue.

3. Experiments on frogs indicate that, when the phosphorylase *a* content of the hind legs has been reduced by electrical stimulation, it is completely restored during a short period of rest.

4. The temporary inactivation of phosphorylase *a* by enzymatic removal of its prosthetic group may represent a regulatory mechanism which would prevent the exhaustion of glycogen stores in fatigue.

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# THE ACTIVITY AND CRYSTALLIZATION OF PHOSPHORYLASE *b*\*

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(Received for publication, December 19, 1944)

It has been found (1) that when phosphorylase *a* is converted to phosphorylase *b* by the PR (or prosthetic group-removing) enzyme in a 0.03 M glycerophosphate-cysteine buffer, the phosphorylase activity in the presence of adenylic acid falls only slightly at the time when the activity without adenylic acid approaches zero. This was taken to indicate that phosphorylases *a* and *b* have the same activity per mg. of protein. Although the experiments have been repeated with the same results, other findings cast doubt on this conclusion.

In Fig. 1 is presented a time curve for the action of PR on phosphorylase *a* and for a control sample of phosphorylase *a* incubated without PR, both in glycerophosphate-cysteine buffer. The control sample when tested with or without adenylic acid shows a slight rise in activity with time, following which the activity remains constant. This rise has been described previously (2) and is probably due to a time factor in the reduction of the enzyme by cysteine in the presence of glycerophosphate. The activity in the sample incubated with PR reaches the same height as the control sample when tested with adenylic acid. Thereafter the activity declines at a rate that would make it about 90 per cent of its original value at the time when the activity without adenylic acid approaches zero; that is, when all of the phosphorylase *a* has been converted to phosphorylase *b*. Similar results have been obtained with trypsin, when it is acting on phosphorylase *a* in a glycerophosphate-cysteine buffer of pH 6.2.

Glycerophosphate seems to exert a stabilizing influence, because without it there occurs a greater decrease in the phosphorylase activity with adenylic acid than was found in the experiments in Fig. 1. This is shown in Fig. 2, which represents an average curve for several experiments. It may be seen that, when conversion is complete, phosphorylase *b* has only 77 per cent of the activity of phosphorylase *a*.

Once all the phosphorylase *a* has been converted to phosphorylase *b*, no further change in phosphorylase *b* activity occurs, even when the incubation is continued with very high concentrations of PR, a fact which has been established in several experiments. Similarly, when phos-

\* This work was supported by a grant from the Rockefeller Foundation.

phorylase *a*. These values are probably too low, because partial inactivation of the enzyme occurs during the slow process of crystallization.

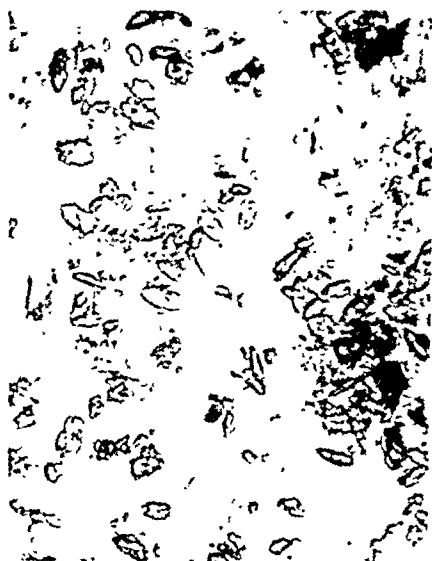


FIG. 3, A

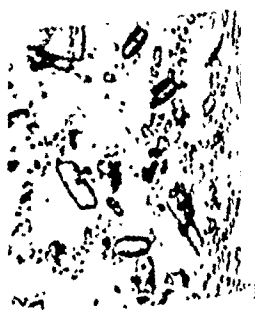


FIG. 3, B

FIG. 3 A, phosphorylase *b* crystallized from ammonium sulfate,  $\times 150$ ; B, phosphorylase *b* recrystallized,  $\times 350$ .

## SUMMARY

1. Activity measurements in reaction mixtures of phosphorylase *a* and PR enzyme in a glycerophosphate-cysteine buffer indicate that upon complete conversion to phosphorylase *b* the latter has about 90 per cent of the catalytic activity of phosphorylase *a*; when the conversion takes place in cysteine without glycerophosphate, phosphorylase *b* shows, on an average, 77 per cent of the activity of phosphorylase *a*. Incubation with PR has no effect on the catalytic activity of phosphorylase *b*. The possibility that some enzyme destruction takes place during conversion of one form into the other has not been excluded.

2. Isolation of phosphorylase *b* from either of the above reaction mixtures by fractionation with ammonium sulfate or by crystallization has not given fractions of greater catalytic activity than was found for the unfractionated material.

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# METHODS FOR THE DETERMINATION OF ALLOXAN, TOGETHER WITH OBSERVATIONS OF CERTAIN PROPERTIES OF ALLOXAN

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During the past few years there has been an increasing interest in the study of the physiological effects of alloxan on plants (1, 2) and animals; rabbits (3-12), rats (7, 8, 13, 14), dogs (5, 15-18), cats (11), mice and frogs (19), man (16).

So far there appears to have been no adequate method available for the measurement of the concentration of alloxan in tissues, blood, or urine. As far as the author is aware, there are only five methods at present described in the literature which have been used for the detection of alloxan. (a) The production of turbidity (colloidal sulfur) when alloxan oxidizes  $H_2S$  (20, 21), (b) the formation of characteristic crystals on reaction of alloxan with copper-pyridine solution (22), (c) the formation of a color on reaction of alloxan with  $\alpha$ -naphthylamine (23), and (d) the precipitation of oxaluramide on treatment of alloxan with  $NH_4CN$  (21) have been used as qualitative tests. (e) The production of a blue color on reaction of ammonium sulfide with alloxan enabled Lieben and Edel (24) to measure alloxan quantitatively. The attempts, however, to stabilize the color have so far been without success. A number of other color reactions of alloxan have been listed in Beilstein (25), but have not been applied to the detection of alloxan in mixtures.

Because the specificity of any method is almost certain to be only a relative matter, the method of choice will depend partly on the nature of interfering substances present, and consequently may vary from one problem to another. For this reason six methods will be described.

## *Factors Governing the Selection of the Most Suitable Method for Determination of Alloxan*

The selection of the method of choice depends upon (1) the concentration of alloxan in the sample, (2) the apparatus available, and (3) the nature and amount of interfering substances in the sample.

The gasometric (I) and titrimetric (II) methods require about 2 mg. of alloxan, but are especially accurate, and offer convenient means of determining the purity of given preparations of alloxan and of determining alloxan present as an impurity in preparations of other oxidation products of uric acid.

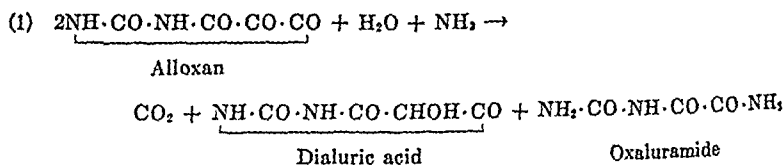
The phosphotungstic acid (III), the diacetylmonoxime (IV), and the *o*-phenylenediamine (V) colorimetric methods are next in sensitivity and measure 0.02 to 0.2 mg. of alloxan. The *o*-phenylenediamine (V) and phosphotungstic acid (III) methods are the simpler of the three colorimetric procedures. The diacetylmonoxime (IV) method is more complicated than the others. It has been included because it measures a product of the KCN reaction (oxaluric acid) not measured by any other method, and may prove useful when relatively high concentrations of reducing substances are present. It is not suited to the determination of alloxan in the presence of relatively high concentrations of urea.

The fluorometric method (VI) is much the most sensitive and measures 0.02 to 0.2  $\gamma$  of alloxan. It is not suited, however, to determination of alloxan in solutions which are fluorescent even before addition of *o*-phenylenediamine.

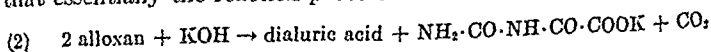
#### *Nature of Reactions Involved in the Determination of Alloxan*

*Reactions of Alloxan with KCN*—The first four methods depend upon the reaction of alloxan with KCN in weakly acid or alkaline solution.

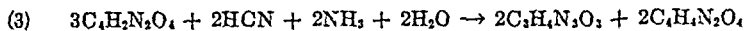
As far as the author is aware, no record of any detailed study of the reaction of alloxan with cyanide has been published since 1860. According to Rosing and Chichkoff (26), reaction of alloxan with ammonium cyanide yields oxalan (oxaluramide). Strecker (27) wrote the equation, which in present day nomenclature is



He pointed out that even earlier (28) it was known that, when potassium instead of ammonium ion is used as the alkali, in the presence of cyanide the salt of oxaluric acid is formed instead of the amide. He observed also that the concentration of cyanide is almost unchanged during the reaction, and concluded that cyanide serves as a catalyst and not as a reactant. We have observed that in the presence of cyanide exactly 1 mole of  $\text{CO}_2$  is produced for every 2 moles of alloxan present. One concludes, therefore, that essentially the reaction proceeds as indicated in Equation 2,



and that not more than an inappreciable fraction proceeds according to the alternative Equation 3 listed by Strecker,



## Alloxan

Oxaluramide    Dialuric acid

in which cyanide is used up and no  $\text{CO}_2$  is produced. Strecker compared the cyanide catalysis of the alloxan reaction with a similar catalysis of benzoin formation from benzaldehyde. Menshutkin (29) prepared what he believed was potassium oxalurate from parabanic acid and  $\text{KOH}$ , but observed that his product differed in properties from the oxalurate obtained by Strecker by action of  $\text{KCN}$  on alloxan. It has been shown (30) that oxidation of pyrimidines (or barbiturates<sup>1</sup>) in alkaline solution (and to a smaller degree in neutral or weakly acid solution) results in ring cleavage and the formation of urea or a monosubstituted urea. The oxidation of alloxan *by alloxan*, as catalyzed by cyanide, appears to fall in line with this scheme.

Liebig (21) used the formation of the sparingly soluble oxalan (oxalamide) on treatment with  $\text{NH}_4\text{OH}$  and  $\text{HCN}$  as proof that alloxan was present in the dialysate of mucous obtained from a patient suffering from "intestinal catarrh."

After action of cyanide on alloxan in the absence of oxygen, the products, dialuric and oxaluric acids, are present in equimolecular amounts. The former, however, being a disubstituted urea, would be expected to react relatively slowly with diacetylmonoxime (32) and, therefore, would contribute relatively little to the color obtained during the heating interval employed.

Information concerning the products of decomposition of alloxan in the presence of cyanide can be summarized as follows: The reaction does not give rise to ammonia or urea. Products known to be produced are (a) 1 mole of  $\text{CO}_2$  per 2 moles of alloxan, and (b) oxaluric acid which reacts with diacetylmonoxime in acid at  $100^\circ$  at the same rate as do other monosubstituted ureas. Since the color produced is the same as that obtained with urea, it is probable that oxaluric acid breaks down rapidly during the heating in acid to form urea and oxalic acid. (c) Dialuric acid. This forms a violet-blue precipitate on addition of excess barium hydroxide (20) and reduces ceric sulfate, ferricyanide, phosphotungstic acid, molybdic acid, Nessler's reagent, and *o*-phenanthroline ferrous complex. The reaction of alloxan in the presence of cyanide (as judged by  $\text{CO}_2$  production) requires about 2 minutes for completion at pH 7 and proceeds more slowly at lower pH. It is almost instantaneous at pH 9. Both the speed of

<sup>1</sup> A method for the determination of barbiturates in blood and urine based on the formation of urea or a monosubstituted derivative on alkaline oxidation (by ultraviolet light and addition of a catalyst such as rose Bengal) and measurement of the resulting urea or derivative by the use of  $\alpha$ -isonitrosopropiophenone (31) or diacetyl-monoxime (32), respectively, is to be published shortly.

conversion of alloxan to alloxanic acid and the speed of decomposition of alloxan, as catalyzed by cyanide, are increased rapidly as the pH rises. Therefore, even when cyanide is present, one would expect part of the alloxan to be converted to alloxanic acid as a side reaction. Actually, however, the amount of alloxanic acid formed must be small and is less than the experimental error of present methods for determining it. The yield of  $\text{CO}_2$  and the reducing material produced in anaerobic decomposition of alloxan by cyanide are so near to the theoretical amounts that very little alloxanic acid can be formed.

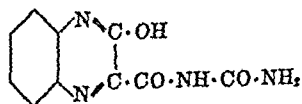
Method I described in the following pages (gasometric) involves the quantitative measurement of the  $\text{CO}_2$  liberated when alloxan, catalyzed by the presence of cyanide, reacts as indicated in Equation 2. At room temperature ( $25^\circ$ ), the reaction is completed almost instantaneously at pH higher than 9. Completion requires 2 minutes at pH 7.0 and, after 5 minutes at pH 5.0, the reaction is only 75 per cent complete.

Method II (titrimetric) involves measurement of the reducing product found when 1 mole of alloxan oxidizes another, according to Equation 2. The dialuric acid is titrated (oxidized) in acid with standard ceric sulfate solution.

Method III measures this reducing substance colorimetrically. Phosphotungstic acid is reduced to give a blue color.

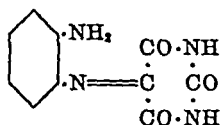
Method IV, another colorimetric method, referred to in previous communications (31, 32), depends on the measurement of another product (oxaluric acid) which reacts much more rapidly than alloxan on heating with diacetylmonoxime in acid solution, yielding a yellow color. The precursor giving rise to this color is not urea, being resistant to attack by urease.

*Reaction with o-Phenylenediamine*—Hinsberg (33), Kühling and Kasselitz (34), and Rudy and Cramer (35) observed that alloxan in neutral solution gives a deep yellow color and a fluorescence in the presence of o-phenylenediamine. Hinsberg (33) proposed a quinoxaline structure for this product.

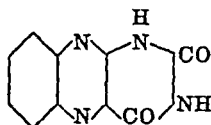


Rudy and Cramer (35), however, observed that N,N-dimethylphenylenediamine (which would not be able to form a quinoxaline derivative) with alloxan yielded a homologous compound with properties similar to those of the product obtained with the unsubstituted o-phenylenediamine. They concluded, therefore, that the product obtained with the di-, mono-, or

unsubstituted *o*-phenylenediamine was an alloxan anil, as had been suggested for the monosubstituted derivative by Kühling and Kaselitz (34).



Rudy and Cramer consider the possibility that the two structures mentioned above exist in equilibrium. It seems unlikely, however, that the alloxan ring, once split, would reform. It is of interest to note that the splitting of the alloxan ring on reduction in weakly acid, neutral, or alkaline solution would be anticipated from the behavior of barbiturates and pyrimidine, and would yield a monosubstituted urea similar in structure to that proposed by Hinsberg. It has been shown by the author that urea or monosubstituted ureas, when heated in acid solution, with diacetylmonoxime (32) or  $\alpha$ -isonitrosopropiophenone (31), yield maximum color much more rapidly than do cyclic disubstituted derivatives of urea, such as alloxan or barbiturates. The product of the reaction of *o*-phenylenediamine with alloxan at pH 4.6, if heated in acid with diacetylmonoxime (32), yields color slightly more rapidly than would be anticipated if the alloxan ring were intact. That is, at least part of the product behaves as a monosubstituted urea. Were it not for the statement that the product of interaction of alloxan with *o*-phenylenediamine can be diazotized, then coupled to an azo dye, Rudy and Cramer would probably have given more consideration to the possibility that the product obtained in neutral or weakly acid solution (in contrast to neutral or weakly alkaline solution) was an alloxazine



as suggested by Kühling (36), or a 3-ring compound with a structure analogous to that which Rudy (37) assigned to the flavins obtained by the action of alloxan on 2,3-diaminopyridines, or 5,6-diaminoquinolines (38).

We have observed that the higher the pH of the phosphate buffer employed, the more rapid is the color development, the lower is the maximum optical density achieved, and the more rapid is the fading of the color. The color produced is not strictly proportional to the concentration of alloxan present, especially with high concentrations of alloxan. The

fluorescence produced, however, is proportional to the concentrations of alloxan employed in the fluorometric procedure.

Method V depends on the formation of this yellow color on reaction with o-phenylenediamine at room temperature and pH 4.5.

Method VI, photofluorometric, is the most sensitive and depends on the fluorescence of the product of this reaction. As has been pointed out by Labes and Freisburger (19), the product is stable in acid solution.

#### *Preparation of Standard Solutions*

As has been shown by Lusini (39) and Labes and Freisburger (19), alloxan in neutral or alkaline solution decomposes rapidly to form alloxanic acid,  $\text{CO}-\text{NH}-\text{CO}-\text{NH}-\text{COH}-\text{COOH}$ . Alloxan solutions are unstable at pH higher than 3.5. Fresh aqueous solutions of alloxan are weakly acid but quickly become more acid as traces of alloxan are converted to the much stronger alloxanic acid. Alloxan concentrations of 1 mg. per cc. or higher quickly acquire a pH of 3.1 or less and are then relatively stable, without addition of other acid. Weaker solutions can be rendered stable by the addition of strong mineral acid. When the pH of the standard is maintained between 2.5 and 3.4, inappreciable loss of alloxan occurs in 24 hours at room temperature even when the concentration of alloxan is as low as 0.5  $\gamma$  per cc. All standard solutions have been prepared by dissolving alloxan in, or diluting its solutions with, 0.002 N  $\text{H}_2\text{SO}_4$ .

Solid preparations of alloxan used for making standard solutions should be stored in an ice box. As pointed out by Gortner (40), crystals stored at room temperature decompose slowly to  $\text{CO}_2$ , urea, oxalic acid, and alloxantin.

#### *Conditions for Preparation of Biological Material for Alloxan Determination*

Alloxan is rapidly destroyed in whole blood, plasma, and urine. The need for precipitation of blood proteins *immediately* (within a matter of seconds) after drawing the samples cannot be overemphasized. Because the method of choice for the precipitation of proteins will vary from one problem to another, no attempt is made in the present outline to prescribe in detail specific precipitation procedures. Certain of our observations, however, may be of assistance to other workers in the selection of suitable procedures. Since glutathione destroys alloxan and is present in blood almost entirely in the red cells, for a study of plasma alloxan the precipitation of plasma proteins without preliminary hemolysis of the cells is advantageous.

Since at the pH of blood alloxan is rapidly destroyed (half life about 4 minutes) even in the absence of glutathione, it is necessary to lower the

pH of the medium as soon as possible (within a matter of seconds) after drawing a sample of blood. Otherwise by the time centrifugation of the blood and separation of the supernatant plasma could be accomplished most of the alloxan present in blood at the time of venipuncture would be destroyed. As has been shown by Herbert, Bourne, and Groen (41), Somogyi filtrates of laked blood contain no glutathione. We have observed, however, that alloxan added to Somogyi filtrates is rapidly destroyed because the pH of these filtrates is not compatible with stability of alloxan. Furthermore, alloxan is precipitated quantitatively by zinc hydroxide. It can be released from the precipitated hydroxide by addition of a solution of  $\text{NaH}_2\text{PO}_4$ .

Filtrates of fresh dog blood prepared by mixing 1 volume of whole blood with 9 volumes of 1.11 per cent sodium tungstate in equivalent (0.073 N) sulfuric acid occasionally contained some glutathione. Folin (42) and Herbert and Bourne (43) simultaneously observed that plasma proteins could be precipitated from whole blood without rupturing the cell membranes. Herbert and Bourne (43) added 1 volume of whole blood to 8 volumes of 3 per cent  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  and followed this with 0.5 volume of 10 per cent sodium tungstate and 0.5 volume of  $\frac{2}{3}$  N sulfuric acid. No glutathione came through into the filtrate. We have modified this procedure by increasing the normality of acid to 0.75 to give a distinctly acid filtrate (pH 3.5) and by mixing the sulfate, tungstate, and sulfuric acid immediately before addition of the blood. The supernatants obtained after immediate centrifugation contain almost no thio groups. The small amount present is probably cysteine and only to a very small extent derived from erythrocytes.

The amount of alloxan which can be recovered after its addition to blood depends largely on the rate at which the blood filtrate is prepared. No matter how rapidly one prepares a filtrate, large losses (due to decomposition of alloxan) are inevitable. Therefore experiments designed to show the degree of recovery of alloxan added to blood appear useless. Record of the results is limited therefore to recovery of alloxan added to blood filtrates.

All procedures outlined below begin on the assumption that the alloxan is in a protein-free solution.

## I. GASOMETRIC METHOD

### *Apparatus*

*Van Slyke-Neill blood gas apparatus* (44).

### *Reagents*

*Cyanide solution*, 0.05 M, 0.65 gm. of KCN in 200 cc. of  $\text{H}_2\text{O}$  (which has been preboiled to remove  $\text{O}_2$ ). Store in an ice box. To protect from



atmospheric  $\text{CO}_2$  and  $\text{O}_2$  it may be stored conveniently in a tonometer over Hg.

*Lactic acid, 1.0 N (approximately).*

### Procedure

2 cc. of solution containing 0.2 to 12.0 mg. of alloxan are delivered from a rubber-tipped stop-cock pipette ((44) p. 125) into the chamber of the Van Slyke-Neill blood gas apparatus. Dissolved oxygen is extracted (with other gases) by lowering the mercury to the 50 cc. mark and shaking the apparatus for 1 minute. The extracted gases are then ejected. 1 cc. of the cyanide solution is introduced with another pipette. The bore of the cock is sealed with Hg and the solutions are mixed. It is important that the pH be 7.0 or higher at this point. After 2 minutes, 0.5 cc. of 1 N lactic acid is introduced, the bore of the cock sealed again with Hg, and the  $\text{CO}_2$  extracted by shaking after lowering the Hg to the 50 cc. mark. The  $p_1$  reading is taken with the gas at the 0.5 cc. mark when the sample contains 3.0 mg. of alloxan or less, at the 2 cc. mark when the sample contains more than 3.0 mg. of alloxan. The temperature is recorded. After adding 0.5 cc. of 5 N NaOH to absorb the  $\text{CO}_2$ , a  $p_2$  reading is taken at the same mark used for the  $p_1$  reading. The procedure followed for measuring the  $\text{CO}_2$  is exactly as described by Van Slyke ((45) p. 277).

The blank must include a correction for the  $\text{CO}_2$  preformed in the solution and reagents. The blank is determined by measuring into the chamber another 2 cc. portion of the alloxan solution, then 0.5 cc. of 1 N lactic acid, and 1 cc. of cyanide solution. No  $\text{CO}_2$  is liberated from alloxan in strongly acid solution even when cyanide is present. The values of  $p_1$  and  $p_2$  are determined as in the analysis. The blank reading  $c$  is calculated as  $c = p_1 - p_2$ .

### Calculation

$$\text{Mg. alloxan in sample} = F \times (p_1 - p_2 - c)$$

where  $F$  is the factor in Table I corresponding to the temperature and volume at which  $p_1$  was read.

The factors in Table I are obtained by multiplying those given by Van Slyke and Sendroy ((46) Table IX,  $S = 3.5$  cc.) by  $3.637 \times 2$ ; 3.637 is the ratio of the molecular weight of alloxan monohydrate to that of  $\text{CO}_2$ . 1 mole of  $\text{CO}_2$  is liberated for every 2 moles of alloxan.

When the sample contains preformed  $\text{CO}_2$  in an amount comparable with, or larger than, that formed by reaction with KCN, it is desirable to alter the procedure as follows, in order to avoid a large blank. To 2 cc. of sample in the blood gas apparatus add 0.5 cc. of 1 N lactic acid, shake

out the  $\text{CO}_2$ , and eject it in the manner outlined by Van Slyke (47) for the determination of urea in whole blood. This extraction is repeated twice. Then add 0.5 cc. of alkaline KCN solution (0.1 M) prepared by dissolving

TABLE I

*Factors by Which Mm. of  $\text{PCO}_2$  Are Multiplied to Give Mg. of Alloxan (As Monohydrate) in Sample Analyzed*

50 cc. apparatus;  $S = 3.5$  cc.

Temperature	$a = 0.5$ cc. $i = 1.036$	$a = 2.0$ cc. $i = 1.017$
°C.		
10	0.01032	0.04051
11	26	25
12	20	01
13	14	0.03977
14	08	54
15	02	32
16	0.00996	09
17	90	0.03888
18	85	68
19	80	47
20	75	27
21	70	07
22	65	0.03787
23	60	67
24	56	48
25	51	29
26	46	11
27	42	0.03694
28	38	76
29	33	59
30	29	42
31	24	26
32	20	10
33	16	0.03595
34	11	79

in 90 cc. of water 0.65 gm. of KCN and 12 cc. of 18 N NaOH from which the carbonate has settled. Then proceed as above.

It is important to have the pH of the mixture pH 7 or higher during the action of the cyanide. Unless oxygen is removed from the alloxan and KCN solutions, the production of  $\text{CO}_2$  is about 4 per cent too high.

## II. TITRIMETRIC METHOD

*Apparatus*

*Reaction tubes.* Thunberg tubes or other tubes of 20 to 40 cc. capacity and capable of being evacuated. Tubes described by Hamilton and Van Slyke (48) for use in determination of amino acids by the ninhydrin- $\text{CO}_2$  method proved convenient.

*Nitrogen tank or carbon dioxide generator.* A 500 cc. Erlenmeyer flask partly filled with dry ice and fitted with a delivery tube serves well.

*Reagents*

*0.05 M potassium cyanide.* 0.65 gm. of KCN in 200 cc. of water.

*Ceric sulfate stock standard, 0.1000 N.* This is prepared and standardized (with Mohr's salt) according to the directions of Miller and Van Slyke (49).

*Dilute working standard, 0.00100 N.* 1 cc. of stock standard and 1 cc. of 18 N  $\text{H}_2\text{SO}_4$  diluted to 100 cc. with water.

*18 N sulfuric acid.* To 50 cc. of water add 50 cc. of concentrated  $\text{H}_2\text{SO}_4$  and make up to 100 cc. with water.

*o-Phenanthroline ferrous complex (50).* 0.025 M stock solution.<sup>2</sup> The working strength, 0.005 M, is prepared by diluting 1 cc. of stock solution to 5 cc. with water.

*Procedure*

1 to 10 cc. of sample (containing 0.5 to 3.0 mg. of alloxan) is placed in the reaction tube. The oxygen of the air in the tube is displaced by holding the tip of the  $\text{CO}_2$  or  $\text{N}_2$  delivery tube about 1 cm. above the level of fluid in the tube. 1 cc. of 0.05 M KCN is then added, the lubricated stopper inserted, and the tube is evacuated at once through the side arm. The pH of the mixture should be above 6.5. After 5 minutes the vacuum is released while the tip of the side arm is held adjacent to the tip of the  $\text{CO}_2$  delivery tube. 1 cc. of 18 N  $\text{H}_2\text{SO}_4$  is added and 1 drop of 0.005 M o-phenanthroline ferrous complex.<sup>3</sup> The mixture is then titrated until the

<sup>2</sup> The stock solution is obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio. For analysis of most solutions this is the most satisfactory indicator. However, it is precipitated in the presence of tungstic acid. Hence, in solutions containing tungstate, o-phenanthroline ferrous complex is replaced by 1 drop of aqueous solution of either alkali-fast green (National Aniline and Chemical Company, Inc.) or of setopaline C, 2 mg. per cc. When either of these alternatives is used, the end-points are compared with the color of a titrated blank held near at hand.

<sup>3</sup> In place of the o-phenanthroline ferrous complex, 0.5 cc. portions of freshly prepared 1 per cent solution of potassium ferricyanide and of 1 per cent ferric chloride have been tried. The ferricyanide is reduced by the product of reaction of alloxan and KCN, and Prussian blue results. The solution remains blue until all the reducing material has been titrated. The use of this alternative as an indicator has

golden brown turns to a very faint blue. A stream of  $\text{CO}_2$  is expelled from a capillary beside the tip of the burette during the titration to maintain an oxygen-free atmosphere in the reaction tube. If air is in contact with the solution during the reaction of cyanide, more ceric sulfate is required to reach the end-point. The error is greater the longer the contact with air. A blank is run with water in place of the KCN solution. The difference in titers is the amount of ceric sulfate equivalent to the alloxan in the samples; 6.25 cc. of 0.001 N ceric sulfate are required for every mg. of alloxan monohydrate.

### Calculation

$$\text{Mg. alloxan monohydrate in sample} = (T_s - T_b) \times 0.16$$

where  $T_s$  = cc. of 0.001 N ceric sulfate required to reach the end-point in the sample.  $T_b$  = cc. of ceric sulfate used by the blank.

### Discussion of Titrimetric Method

The presence of the slowly oxidizable glutathione or cysteine in a sample destroys the sharpness of the end-point. Consequently the end-points observed during the titration of urines and Folin-Wu filtrates (which contain glutathione) are not sharp. It is especially important in such cases to titrate the blank at the same rate as the KCN-treated sample. End-points obtained with modified Herbert and Bourne or Somogyi filtrates are sharp.

Hill (51) has shown that dialuric acid in acid solution is readily oxidized to alloxan.<sup>4</sup> This oxidation can be accomplished by atmospheric oxygen at room temperature as well as by oxidizing agents such as ceric sulfate. This leads to a source of error in the titrimetric method unless precautions to exclude oxygen are observed. If atmospheric oxygen is in contact with the alloxan solution during or following the action of cyanide, part of the dialuric acid formed will be oxidized to alloxan. Action of cyanide on this regenerated alloxan would be expected to yield only 50 per cent of the dialuric acid from which this alloxan was derived. After acidification of

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the slight advantage that a greater depth of color is obtained and that the reagents are commonly available. It has the disadvantages that the end-point is (1) yellow rather than almost colorless, and (2) is not as sharp as when o-phenanthroline ferrous complex is used.

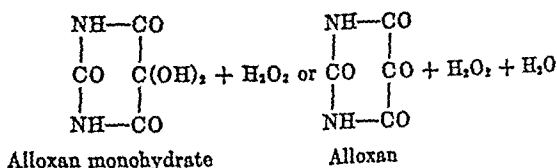
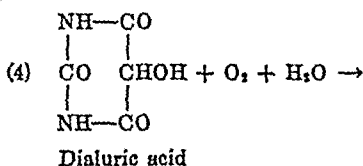
<sup>4</sup> Labes and Freisburger (19) indicate that a solution of murexide behaves as a mixture of alloxan, alloxantin, and ammonia. Richardson (52, 53) points out that in solution alloxantin appears to dissociate to a large extent to dialuric acid and alloxan. Therefore murexide or alloxantin will give reactions for dialuric acid. Furthermore, in any reaction involving reduction of alloxan the immediate product is dialuric acid. If an equivalent or more of alloxan remains, alloxantin is formed. If both ammonia and an excess of alloxan are present, murexide results.

the solution, oxidation of dialuric acid by atmospheric oxygen continue. The reconversion of alloxan to dialuric acid by cyanide is, however, almost completely inhibited by the high concentration of  $H^+$  ion. One would therefore expect the titer to be low by an amount equivalent to the dialuric acid oxidized by air during titration plus half the dialuric acid oxidized before acidification.

Actually, however, for reasons considered below, contact of the reaction mixture with air before acidification causes the titer to be too high. The longer this contact, the greater the error introduced. The positive error amounts to about 1 per cent for every minute of contact with air.

For these reasons the reaction with cyanide is best conducted *in vacuo* and acidification and titration in a  $CO_2$  atmosphere follow immediately after release of the vacuum. When the procedure is followed as outlined, the titers are independent of the duration of action of cyanide and check within 0.5 per cent.

The reason for this increase in titer on contact with oxygen was investigated. It appears that in neutral or alkaline solution dialuric acid in the presence of oxygen and cyanide yields alloxan and a peroxide, probably hydrogen peroxide,<sup>5</sup> as indicated by Equation 4.



Hydrogen peroxide reduces ceric sulfate. Therefore 1 equivalent of dialuric acid gives rise to 1 equivalent of peroxide and 1 equivalent of alloxan (which latter in neutral cyanide yields, according to Equation 2, 0.5 equivalent of dialuric acid). Hence for each complete oxidation-reduction cycle 1 equivalent of dialuric acid yields 1.5 equivalents of material capable of reducing ceric sulfate, and preliminary action of atmospheric  $O_2$  increases the amount of ceric sulfate used in subsequent titration, instead of diminishing it, as might be expected.

<sup>5</sup> Such a reaction has been postulated by Richardson (53) on the basis of the amount of oxygen utilized in the oxidation which occurred in the presence of cyanide.

The formation of peroxide was demonstrated by the chemiluminescence (54) produced in a fresh mixture of a 0.1 per cent solution of 3-aminophthalhydrazide (luminol) in 1 per cent  $\text{Na}_2\text{CO}_3$ , and 0.00005 M hemin in 1 per cent aqueous  $\text{Na}_2\text{CO}_3$ . On addition of either cyanide or alloxan no luminescence was observed even on shaking the mixture in air. On addition of both cyanide and alloxan luminescence appeared at once and was intensified by shaking the mixture in air. A mixture of alloxan and KCN which had stood in contact with air for several hours gave a very intense luminescence with this reagent.

### III. PHOTOMETRIC METHOD WITH PHOSPHOTUNGSTIC ACID

#### *Apparatus*

*Photometer or colorimeter.* A Coleman junior spectrophotometer has proved satisfactory. Cylindrical cuvettes with an internal diameter of 16 mm. were used.

#### *Reagents*

*0.002 N sulfuric acid* (approximate). 0.1 cc. of concentrated sulfuric acid added to 1800 cc. of water.

*Stock standard solution of alloxan.* 100 mg. of monohydrate in 100 cc. of 0.002 N  $\text{H}_2\text{SO}_4$ .

*Working standard, 0.02 mg. per cc.* 2 cc. of stock standard solution diluted to 100 cc. with 0.002 N  $\text{H}_2\text{SO}_4$ . Fresh daily.

*Phosphotungstic acid.* Prepared according to the directions of Kern and Stransky (55). To 50 gm. of reagent grade sodium tungstate add 400 cc. of water and 40 cc. of syrupy phosphoric acid. Reflux for 2 hours; make up to 500 cc. with water and store in a brown bottle.

*0.67 M dibasic sodium phosphate.* 240 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  to 1 liter with water.

*Potassium cyanide solution, 0.5 M.* 6.5 gm. of KCN in 200 cc. of water. Store in an ice box.

*Reagent mixture with KCN.* Enough of this reagent for the series is prepared *immediately* before use by mixing in the following order: 3 volumes of 0.67 M dibasic sodium phosphate,<sup>\*</sup> 0.05 volume of 0.5 M KCN, and 1 volume of phosphotungstic acid reagent. Addition of the phosphotungstic acid to the alkaline buffer should precede the use of the reagent by not more than 1 minute. The longer the mixture stands before use the less will be the intensity of the color obtained.

*Reagent mixture without KCN.* Prepared exactly as above except that

<sup>\*</sup> Substitution of potassium salt would result in precipitation of potassium phosphotungstate in the reagent mixture. The concentration of  $\text{K}^+$  from the KCN is not sufficient to cause precipitation.

0.05 volume of water replaces the cyanide solution. This too is prepared *immediately* before use.

### *Procedure*

To a 5 cc. sample solution containing 0.015 to 0.15 mg. of alloxan and also to 5 cc. of water add 1 cc. portions of the reagent mixture with cyanide and mix. To other 5 cc. aliquots of sample and water add 1 cc. of the reagent mixture without cyanide. Standards made up to 5 cc. with water and containing 1, 2, or 3 cc. of dilute working standard are treated similarly. Allow the color to develop for 30 minutes in the dark and then read the optical density of the samples against the water blank in a photoelectric colorimeter set at wave-length 700 m $\mu$ . Alternatively the optical density in the samples can be read against a standard in a visual colorimeter. The color in the sample to which reagent without KCN was added is due to reduction of phosphotungstic acid by substances other than alloxan. The difference between the optical densities obtained with and without cyanide is proportional to the alloxan present (see Fig. 1).

### *Conditions Governing the Development of the Color with Phosphotungstic Acid*

The mixture of phosphotungstic acid and dibasic phosphate with or without cyanide is slightly acid. The more nearly neutral it is made the faster the color develops from reduction of  $W^{++++}$  and the more rapidly does the color fade. Under the conditions specified the color development is complete in 20 minutes, after which time loss of 1 per cent of the color occurs every hour.

Best results are obtained when all three components of the reagent are added together in a single solution. If addition of cyanide should precede that of the other two components by 5 minutes, almost no color would be obtained. As has been pointed out already, the alloxan is unstable in alkali or at a pH near neutrality; therefore the basic phosphate must not precede the other components.

### IV. PHOTOMETRIC METHOD WITH DIACETYLMONOXIME

This method is not recommended for visual colorimetry or for use when the concentration of urea is greater than that of the alloxan. Preliminary removal of urea from solution by urease is not practical, since alloxan is rapidly destroyed at pH values suitable for action of urease.

### *Apparatus*

*Photometer or colorimeter.* Cylindrical cuvettes with an inside diameter of 16 mm. have been used for this method.

*Test-tubes* of uniform shape and about 30 cc. capacity for heating the solutions during color development. Tubes  $20 \times 150$  mm. have proved satisfactory. Each tube is fitted with a rubber stopper through which passes a glass capillary (31).

### Reagents

*Diacetylmonoxime*, 3 per cent in  $H_2O$ .

*Potassium cyanide crystals* (reagent grade).

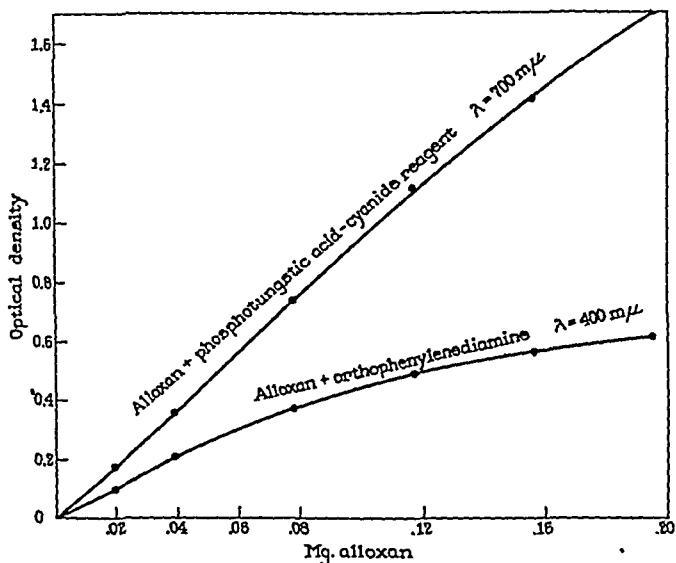


FIG. 1. Proportionality of the optical density to the amount of alloxan in the sample in the phosphotungstic acid and *o*-phenylenediamine methods. 5 cc. of solution + 1 cc. of reagent. The reactions were conducted under the conditions prescribed in the procedures.

*Standard solution of alloxan* containing the equivalent of 0.04 mg. of alloxan monohydrate per cc. 4 mg. of alloxan are dissolved in 100 cc. of 0.002 N  $H_2SO_4$ .

*Anhydrous potassium carbonate*.

*Acid mixture*. Mix 1 volume of reagent sulfuric acid with 3 volumes of reagent phosphoric acid and 1 volume of water (31).

### Procedure

Duplicate portions of 5 cc. each of the alloxan solution (containing 0.025 to 0.25 mg. of alloxan) are pipetted into the tubes to be used for the subsequent heating.



Eight tubes with standards are prepared by measuring duplicate portions each of 1, 2, 4, and 5 cc. of stock standard solution with similar tubes. Unknowns and standards are then each made up to 7 cc. by addition of water.

In one tube of each pair (standards and unknowns) is placed a small crystal of KCN (about 0.5 mg.), and if the solutions are acid small portions (about 5 mg.) of anhydrous  $K_2CO_3$  are added until effervescence ceases. (Avoid breathing fumes of the HCN!) The  $CO_2$  in the tube is displaced by blowing air through a pipette held half-way down the inside of the tube. The liquid is mixed thoroughly. After 20 minutes the conversion of alloxan to dialuric acid and oxaluric acid will be complete. 7 cc. of water in another tube serve as a blank. It is unnecessary to prepare a blank to which cyanide has been added. Then to each tube, unknown, standard, and blank (both with and without KCN), are added 5 cc. of the phosphoric-sulfuric acid mixture and 0.5 cc. of 3 per cent diacetylmonoxime reagent. Mix the solutions, stopper, and then heat all the tubes exactly 15 minutes in a boiling water bath. Hereafter protect the tubes from light. Cool the tubes in a cold water bath and read the optical density in a spectrophotometer set to a wave-length of 470  $m\mu$ . If the tubes are kept in absolute darkness after heating, they may be read at any time after they are cool up to 12 to 24 hours.

The samples (both with and without KCN) are read against the reagent blank set at zero optical density.<sup>7</sup> The standards to which KCN was added are read against the corresponding standard to which no KCN was added set to zero optical density. Plot the optical densities read for each standard against the corresponding mg. of alloxan monohydrate.

### Calculation

From the curve so obtained read off the mg. of alloxan equivalent of (a) the optical density of the sample tube treated with cyanide (A), and (b) the corresponding sample tube untreated with KCN (B).

$$\text{Mg. alloxan monohydrate in aliquot of solution heated} = A - B$$

### Discussion of Photometric Method with Diacetylmonoxime

Subtraction of the alloxan equivalent of the color formed in solutions not treated with KCN corrects for the small amount of color formed from

<sup>7</sup> Frequently the color obtained with unknowns is so great, even in the absence of cyanide, that the instrument containing such a solution cannot be adjusted to read zero optical density. Therefore a reagent blank, rather than an unknown not treated with cyanide, is used as a blank against which cyanide-treated unknowns are read. In case of the standards, however, it is convenient to read directly the increase in color resulting from the cyanide treatment.

alloxan itself in the absence of cyanide, as well as for color due to substances other than alloxan. Since the points from which the standard curve is made are obtained by reading each cyanide-treated standard against the corresponding standard not treated with cyanide, the method depends on the measure of the *increase* in color production resulting from the presence of KCN in the solution.

Under the conditions outlined in Methods III and IV, in the presence of cyanide essentially all of the alloxan is converted to oxaluric acid by operation of the cycle indicated by Equations 2 and 4. If the determination were conducted anaerobically, the reaction indicated in Equation 4 could not take place and only half as much oxaluric acid would be formed. Although hydrogen peroxide decreases slightly the amount of color obtained with phosphotungstic acid, the intensity of the colors developed in Methods III and IV is not affected appreciably by the amount of peroxide which could be formed by the reaction expressed by Equation 4.

#### V. PHOTOMETRIC METHOD WITH *o*-PHENYLENEDIAMINE

##### *Apparatus*

*Photometer or colorimeter.* Cylindrical cuvettes with an inside diameter of 16 mm. have been used.

##### *Reagents*

*Molar sodium dihydrogen phosphate*, 138 gm. of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  made up to 1 liter with water.

*o-Phenylenediamine stock solution.* 50 mg. are dissolved in 100 cc. of glycerol, placed in a brown bottle, and stored in the dark. This solution remains colorless and active for at least 6 months.

*Working solution.* 5 cc. are diluted to 10 cc. with molar  $\text{NaH}_2\text{PO}_4$  within an hour before use.

*Stock standard solution of alloxan*, 1 mg. of alloxan monohydrate per cc. 100 mg. of alloxan monohydrate are dissolved in 100 cc. of 0.002 N  $\text{H}_2\text{SO}_4$ . This is prepared fresh weekly or is stored frozen (in solid  $\text{CO}_2$ ).

*Working standard solution of alloxan*, 0.04 mg. per cc. This is made fresh each day. 2 cc. of stock standard are diluted to 50 cc. with 0.002 N  $\text{H}_2\text{SO}_4$ .

##### *Procedure*

To 5 cc. portions of sample containing 0.02 to 0.2 mg. of alloxan, and likewise to 5 cc. portions of  $\text{H}_2\text{O}$  (for the blank), and of alloxan standards (containing 2, 4, and 5 cc. of working standard made up to 5 cc. with water), add 1.0 cc. portions of the "working solution" of phenylenediamine and mix. After 20 minutes read the optical densities of the standards and samples

against the blank set at zero optical density at a wave-length of 390  $m\mu$ . A wave-length setting of 400  $m\mu$  gives satisfactory results and can be used when the instrument does not permit a shorter wave-length setting.

### Calculation

*Photometer Readings*—The optical densities of the standards are plotted against the corresponding weight of alloxan present. The mg. of alloxan in the sample is read from this curve. Since Beer's law is not followed accurately, standards for use in visual colorimetry must be almost equal in alloxan concentration to the unknowns. See Fig. 1.

### Visual Colorimetric Readings—

$$\text{Mg. alloxan in sample} = \frac{D_{sa}}{D_{st}} \times \text{mg. alloxan in standard}$$

where  $D_{sa}$  = reading of sample and  $D_{st}$  = reading of standard.

## VI. PHOTOFLUOROMETRIC METHOD WITH *o*-PHENYLENEDIAMINE

### Apparatus

*Photofluorometer.* A Coleman model 12A is satisfactory.

*Filters* (primary) Corning No. 5113, (secondary) Corning No. 3381.

### Reagents

*Standard alloxan, 0.0002 mg. per cent.* This is prepared fresh by diluting the working standard used in the previous photometric procedure. 1 cc. of standard containing 0.04 mg. per cc. is diluted to 200 cc. with 0.002  $N$   $H_2SO_4$ .

*Molar phosphate buffer and o-phenylenediamine stock* are prepared exactly as in the previous method.

*Working solution of o-phenylenediamine reagent* is prepared by diluting 1 part of stock reagent with 10 parts of molar sodium dihydrogen phosphate.

### Procedure

To 5 cc. of sample add 0.5 cc. of "working solution" of *o*-phenylenediamine. Set the tubes in a dark place. After 1 hour read in a fluorometer against a reagent blank and compare with a standard solution of approximately the same strength as the sample.

### Calculation

$$\text{Mg. alloxan in sample} = \frac{\text{reading of sample}}{\text{reading of standard}} \times \text{mg. alloxan in standard}$$

The diamine reagent on exposure to light, especially ultraviolet or blue light, turns yellow and develops marked fluorescence of its own. Therefore

the blank due to the reagent is markedly increased if the tubes are allowed to stand close to and in the path of light from the fluorometer before the readings are taken.

#### SPECIFICITY OF THE METHODS

*I. Gasometric Method*—Alloxan and compounds which dissociate into alloxan (e.g., *alloxantin*) yield  $\text{CO}_2$  on treatment with cyanide. In the absence of oxygen 0.5 mole of  $\text{CO}_2$  is liberated for every mole of alloxan present. The yield of  $\text{CO}_2$  from *alloxantin* corresponds to the amount of alloxan combined in it. That is, 1 mole of *alloxantin* is equivalent to 1 of alloxan and 1 of dialuric acid and yields 0.5 mole of  $\text{CO}_2$ . Weight for weight *alloxantin* yields approximately half as much  $\text{CO}_2$  as does alloxan.

*Ninhydrin*, because of its structural similarity, also yields  $\text{CO}_2$  in the presence of cyanide. The reaction, however, is much slower and at  $25^\circ$  and pH 10 the reaction is only 10 per cent complete after 2 minutes. Under anaerobic conditions *dialuric acid* (prepared according to the method of Biltz and Damm (56)) yields no  $\text{CO}_2$ . Likewise, *parabanic acid*, *allantoin*, *cysteine*, *glutathione*, *esculin*, and *caffeine* yield no  $\text{CO}_2$  in the presence of cyanide. *Ascorbic acid* and *uric acid* in cyanide and under the prescribed conditions of analysis yield less than 5 per cent of the  $\text{CO}_2$  obtained with equivalent amounts of alloxan.

*II. Titrimetric Method*—This method, like the phosphotungstic acid colorimetric method, depends for its specificity on the increase in reducing action resulting from treatment with cyanide. Although a wide variety of substances reduce ceric sulfate, only *alloxan*, *alloxantin*, and *ninhydrin* are known to have their reducing power towards ceric sulfate increased by treatment with cyanide. Neither alloxan nor *ninhydrin* reduces ceric sulfate in the absence of cyanide. On treatment with cyanide 1 mole of alloxan yields 0.5 mole of dialuric acid, which reduces ceric sulfate. In the same manner the mole of alloxan combined in 1 mole of *alloxantin* yields an additional 0.5 mole of dialuric acid on treatment with cyanide. *Ninhydrin* on addition of cyanide likewise gives rise to reducing material.

*Parabanic acid*, *caffeine*, *oxaluric acid* (prepared according to the method of Biltz and Topp (57)), and *allantoin* do not reduce ceric sulfate. *Dialuric acid* (and therefore also *alloxantin*), *uric acid*, *esculin*,<sup>a</sup> and *ascorbic acid* reduce ceric sulfate rapidly whether cyanide is present or not. Likewise reduced *glutathione*, *cysteine*, and *ergothioneine* also reduce ceric sulfate, although they do so very slowly. The end-points obtained with solutions containing these three substances are therefore not sharp. Except for

<sup>a</sup> Esculin forms a transient deep blue color on treatment with ceric sulfate. This color changes to a golden brown (more rapidly in the presence than in the absence of cyanide).

alloxantin, the reducing power of the last seven compounds mentioned, towards ceric sulfate, is not altered by treatment with cyanide.

*III. Photometric Method with Phosphotungstic Acid*—Under the conditions outlined for the procedure, alloxan and ninhydrin do not reduce phosphotungstic acid in the absence of cyanide. Dialuric acid<sup>9</sup> and compounds (such as alloxantin) which dissociate into dialuric acid do reduce phosphotungstic acid, either in the presence or the absence of cyanide. Cyanide does not intensify the color produced from dialuric acid, but does intensify the color obtained with alloxantin (because alloxan is one of the dissociation products of alloxantin). Ascorbic acid rapidly reduces phosphotungstic acid even in the absence of cyanide. Addition of cyanide, however, doubles the intensity of the blue color obtained with ascorbic acid. Weakly acid solutions of glutathione and cysteine also reduce phosphotungstic acid rapidly in the absence of cyanide. However, on addition of cyanide the reduction of phosphotungstic acid by glutathione and cysteine is decreased to less than 7 per cent of its former level. Ergothioneine reduces phosphotungstate slowly both in the absence and especially in the presence of cyanide. Presumably the addition of cyanide decreases the rate of reduction of phosphotungstic acid by thiol groups but increases the rate of reduction by other parts of the molecule. At the prescribed pH, uric acid causes very slight reduction of phosphotungstic acid either in the presence or absence of cyanide. Parabanic acid, caffeine, esculin, oxaluric acid, and allantoin yield no color either with or without cyanide.

To summarize: The blue color obtained with phosphotungstic acid is increased by addition of cyanide in the presence of alloxan, alloxantin, ninhydrin, and ascorbic acid. An almost inappreciable increase is caused in the presence of cyanide by uric acid and ergothioneine. The color given by glutathione and cysteine is decreased when cyanide is present.

In the reaction of alloxan with thiol groups 1 mole of alloxan yields a mole of dialuric acid, whereas with cyanide 1 mole of alloxan yields only 0.5 mole of dialuric acid. Therefore if alloxan (concentration =  $A$ ) is added to a solution containing a smaller concentration of glutathione or cysteine (thiol concentration =  $T$ ), the color obtained with phosphotungstic acid in the presence of cyanide,  $\frac{1}{2}(A - T) + T$ , is greater than that which would

<sup>9</sup> Dilute solutions of dialuric acid are very quickly oxidized to alloxan by atmospheric oxygen unless precautions are observed to keep air from the system. After oxidation to alloxan they no longer reduce phosphotungstic acid unless cyanide is added. Addition of cyanide to the oxidized dialuric acid would yield only 50 per cent as much dialuric acid as was present before oxidation. Solutions of dialuric acid are therefore prepared in water from which dissolved oxygen has been removed by evacuation. An atmosphere of CO<sub>2</sub>, H<sub>2</sub>, or N<sub>2</sub> is then maintained above the water before and after addition of the dialuric acid.

be obtained with the same amount of alloxan in the absence of thiol groups ( $\frac{1}{2}A$ ). On subtraction of the blank  $T$ , obtained in the absence of cyanide, the difference,  $\frac{1}{2}(A - T)$ , is equivalent to the amount of alloxan actually remaining in solution after reaction with the thiol groups and is therefore equivalent to less than the amount added. If  $T$  is greater than  $A$ , then no alloxan is left in solution and the negative difference obtained on subtracting the blank corresponds to the amount of thiol group left unattached by alloxan.

IV. *Colorimetric Method with Diacetylmonoxime*—The specificity of color formation with diacetylmonoxime in acid has been discussed in a previous communication (32). The specificity of the method as applied to alloxan depends on the formation of a monosubstituted urea when alloxan decomposes in the presence of cyanide. Only *alloxan and compounds dissociating to give alloxan* show an increase in color production on heating in acid with diacetylmonoxime as a result of treatment with cyanide. This method is tedious and is not recommended for routine determination if the sample contains urea. It is included since it may prove useful when run in conjunction with one of the other methods in testing specificity.

V. *Photometric Method with o-Phenylenediamine*—Formation of a yellow color occurs in the presence of *alloxan* and *alloxantin*. *Ninhydrin* yields a yellow precipitate. Under the prescribed conditions of analysis *ascorbic acid* gives inappreciable color but if the mixture of diamine and ascorbic acid is exposed to daylight or especially to ultraviolet light, it yields a yellow color much faster than does the reagent blank.<sup>10</sup> No color was produced with *glutathione*, *cysteine*, *parabanic acid*, *uric acid*, *allantoin*, *oxaluric acid*, *caffeine*, or *esculin*.

VI. *Fluorometric Method with o-Phenylenediamine*—*Alloxan* and *compounds which dissociate into alloxan* yield a green fluorescence with *o*-phenylenediamine. *Ninhydrin*, which is itself fluorescent, yields a product with a stronger fluorescence. *Ascorbic acid* yields a product having a blue fluorescence.<sup>10</sup> No fluorescence product is obtained with *parabanic acid*, *uric acid*, *cysteine*, *glutathione*, *ergothioneine*, *allantoin*, or *caffeine*.

### Results

Table II shows the recovery of alloxan added to modified Herbert and Bourne, Folin-Wu, and Somogyi filtrates of dog whole blood as determined by the two simplest colorimetric methods.

<sup>10</sup> Methods for the determination of ascorbic acid in blood and urine based on the formation of a blue fluorescence on reaction in the dark with *o*-phenylenediamine and of a yellow color on exposure of the product to ultraviolet light are being developed and will be published later. Dehydroascorbic acid (structurally related to alloxan) is the product measured.

## DISCUSSION

*Observations Concerning Tests for Purity of Alloxan Preparations*

Determination of the nitrogen content of alloxan samples by the micro-Kjeldahl method of Van Slyke and Kugel (58) leads to values which are 0 to 10 per cent lower than those obtained when other organic matter (e.g., 30 mg. of glucose) is added to each sample before the acid digestion.

Because several impurities frequently present in commercial preparations of alloxan contain almost the same per cent nitrogen, a sample which shows theoretical nitrogen content by Kjeldahl analysis is not necessarily pure.

Pure alloxan treated with excess of  $\text{Ba}(\text{OH})_2$  does not yield a purple color. Preparations containing decomposition products such as isodialuric (59) and dialuric acid (20) yield a purple color with excess  $\text{Ba}(\text{OH})_2$ .

TABLE II

*Recovery of Alloxan Added to Blood Filtrates. Phosphotungstic Acid and o-Phenylenediamine Methods*

1 cc. of solution containing 0.20 mg. of alloxan was added to 5 cc. of 1:10 filtrate of whole dog blood. The mixtures were analyzed as outlined in the procedures.

Filtrate	Alloxan recovered by phosphotungstic acid method	Alloxan recovered by o-phenylenediamine method
	per cent	per cent
Folin-Wu.....	93	97
Modified Herbert and Bourne.....	96	96
Somogyi*.....	98	99

\* Unless analyses are done *immediately* after addition of alloxan, there is marked loss of alloxan due to conversion to alloxanic acid at the pH of the Somogyi filtrates. Alloxan is precipitated quantitatively by Somogyi reagents.

This color sometimes is intensified on addition of  $\text{NaOH}$ , although  $\text{NaOH}$  without  $\text{Ba}(\text{OH})_2$  yields almost no color.

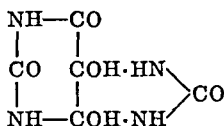
Pure alloxan (prior to treatment with cyanide) does not reduce ceric sulfate. Impure preparations reduce it even without addition of cyanide. Therefore the difference obtained in the titer of an aliquot of sample and of water, when no cyanide is used, is a measure of the alloxantin<sup>4</sup> present.

*Reaction of Alloxan with Blood Constituents*

*Urea*—Mulder (60) observed that when a solution containing equimolecular concentrations of alloxan and urea is evaporated slowly (over a 3 day period) star-shaped groups of crystals of alluranic acid are formed. The product is weakly acid, gives a negative test for alloxan with ferrous sulfate and ammonia, is only slightly soluble in cold water, and therefore differs in this respect from alloxanic acid-urea. Alluranic acid is very

soluble in dilute alkali ( $K_2CO_3$ ) and is slowly decomposed by  $NH_4OH$ , yielding a product differing from dialuric acid. It is precipitated by acids from a fresh solution and can be boiled without decomposition in dilute acetic acid but not in dilute hydrochloric acid. It is unaffected by treatment with  $H_2S$  (60).

Biltz and Heyn (61) pointed out that Mulder's compound could be obtained as flat prisms with 0.5 mole of water of crystallization when the formation took place over  $CaO$ . A solution of alloxan and urea dried over  $H_2SO_4$  yielded only crystals of the unchanged solutes. Biltz and Heyn assigned the formula



We have observed that on mixing concentrated solutions of alloxan (1.6 gm.) and urea (0.6 gm.) in equimolecular amounts either in water or in 0.1 N  $H_2SO_4$  white crystals separate after 30 to 45 seconds. The product is much less soluble than alloxan or urea. A dilute aqueous solution of the product behaves as though it contained only the two reactants. Urease slowly removes urea from a solution of the product and phosphotungstic acid is reduced by the product in the presence of cyanide. In this respect it differs from ninhydrin ureide. The product is therefore either a slightly dissociated alloxan salt of urea or a hydrated ureide which is easily hydrolyzed to alloxan and urea. It is of interest in this connection that Van Slyke and Hamilton (62) showed that urea and ninhydrin (which is structurally related to alloxan) combine readily to form ninhydrin ureide. As pointed out by Mulder (60) alloxan will not remain unchanged for long when it is present in solutions containing urea (as for example in blood or urine). However, we observe that solutions of alloxan containing an equivalent of urea after standing 12 hours at room temperature cause the same amount of reduction of phosphotungstic acid-cyanide reagent as is caused by a fresh mixture of alloxan and urea or by alloxan in the absence of urea.

*Amino Acids*—Alloxan reacts with amino acids (except proline) at pH near 7.0 to give purpuric acid salt (63) which is colored in neutral or alkaline solution (19), an aldehyde containing one less  $CH_2$  than the amino acid (64) and  $CO_2$  and  $NH_3$ . Wieland and Bergel (65) note that the reaction is catalyzed by Pd black. Lieben and Edel (24) concluded that color formation on interaction of alloxan with amino acid (or  $NH_3$ ) was unsatisfactory as a measure of either alloxan or amino acids (or  $NH_3$ ) because (a) the color faded rapidly, (b) the method was not sufficiently



sensitive (especially with  $\text{NH}_3$ ), (c) the color was not proportional either to the alloxan or to the amino acid (or ammonia) present. This reaction with alloxan is analogous to that of ninhydrin with amino acids. It is interesting that the same conclusions have been reached with respect to ninhydrin as a color reagent for measuring amino acids (66, 67) and ammonia<sup>11</sup> as Lieben and Edel reached with alloxan. Lieben and Edel (24) pointed out that alloxan reacts more easily with the SH groups of amino acids and proteins than with the  $\alpha\text{-NH}_2$  groups and that the color obtained when alloxan reacts with proteins or amino acids containing SH groups is due largely to oxidation of thiol groups. Therefore alloxan injected into circulating blood is converted rapidly (a) to alloxanic acid by the alkali, (b) to alloxantin,<sup>4</sup> thence to dialuric acid by the thiol groups of plasma proteins circulating cysteine and any small amount of glutathione which may be present in plasma; a slower reaction with the  $\alpha\text{-NH}_2$  groups of circulating amino acids with formation of dialuric acid (or alloxantin or purpuric acid) would be anticipated. (c) Part (although a small part) of the alloxan probably combines with urea.

*Interpretation of Certain Biological Effects of Alloxan in the Light of Its Chemical Properties*

Bernheim (68) states it is difficult to decide whether the inhibition by cyanide of the 10-fold acceleration by alloxan of  $\text{O}_2$  uptake of slices of guinea pig liver acting on ethyl alcohol is due to the inhibition of a cyanide-sensitive system through which alloxan must act, or whether it is due to a combination of cyanide with alloxan, thus inactivating it directly. It would seem that decomposition of alloxan in the presence of cyanide is sufficient to account for the cyanide effect observed by Bernheim even though the system may be sensitive to cyanide on other accounts.

The fact that reduced glutathione partly counteracts the inhibition by alloxan of the formation of Robison and Cori esters (69) and that alloxan retards fermentation by yeast cells (2) is not surprising in view of the destruction of thiol groups by alloxan reported by Labes and Freisburger (19). Addition of alloxan to filtrates of whole blood containing glutathione, or to glutathione alone, results in the formation of dialuric acid.<sup>4,9</sup> The product of this reaction has been identified by its reducing power and by formation of a deep violet-purple color on addition of excess baryta and by its reconversion on contact with  $\text{O}_2$  to form alloxan.

The possibility that circulating blood normally contains small amounts of alloxan should not be overlooked, especially in view of the report by Ascoli and Izar (70) and Preti (71) that dog liver or blood contains an enzyme system capable of splitting uric acid to dialuric acid in the presence of oxygen and of synthesizing uric acid from dialuric acid in the absence of

<sup>11</sup> Unpublished data by D. D. Van Slyke and R. M. Archibald.

oxygen. Dog liver perfused with normal blood destroyed uric acid. When, however, the blood was saturated with  $\text{CO}_2$ , uric acid reappeared (70). The marked rise in the blood level of uric acid which is observed in dogs in which severe shock is induced (72) might be explained in part by *in vivo* operation of this enzyme system.

Lang (73) reported a single case of a patient who excreted either alloxan or a precursor of alloxan in urine. This sugar-free urine, which was yellow on voiding, turned reddish blue and alkaline on standing several hours. This change to alkalinity and the resulting odor of ammonia could be accounted for by the oxidation on contact with air, of the relatively strong dialuric acid to the weaker alloxan. This latter, once formed, would react with dialuric acid and ammonium ion to form the colored murexide. The possibility that the rapid rise in pH of the urine and the formation of alloxan or precursors were the result of bacterial action on urea and uric acid, respectively, should not be overlooked. Such a rapid increase in alkalinity, however, as that reported would be bacterial in origin only if there was gross infection, and the reported absence of leucocytes and erythrocytes leads one to believe that Lang's patient may have had an unusual metabolic anomaly rather than cystitis or pyelitis.

If dialuric acid is formed in blood, it would not be surprising if minute amounts of alloxan were formed by action of molecular oxygen carried by the hemoglobin.<sup>12</sup> As indicated, however, in previous communications (31, 32), if alloxan is present in plasma, its concentration is inappreciable. Application of the most sensitive method (fluorometric) to filtrates of normal dog and human blood prepared rapidly by the modified Herbert and Bourne method indicates that the concentration of alloxan in normal plasma is less than 0.02 mg. per 100 cc.

Since this manuscript went to press, the author has been informed by Leech and Bailey that they will publish shortly (74) two methods for the determination of alloxan in blood. Their findings with respect to the instability of alloxan in plasma have been confirmed, in part, by the author. The writer deeply appreciates the kindness of these workers in loaning a copy of their manuscript prior to its appearance in print.

The author is indebted to Dr. D. D. Van Slyke for help in preparing the manuscript. Miss E. Stroh, Miss P. Ortiz, Mr. J. Bronner, and Mr. A. Despopoulas rendered valuable technical assistance during the development of the methods.

<sup>12</sup> No significant change in the blood sugar level of dogs has been observed 2, 5, and 48 hours after the intravenous injection (150 mg. per kilo) of freshly prepared dialurate (56), or after repeated doses of half as much ninhydrin. The observation with dialurate confirms that of Jacobs (3) with rabbits and of Goldner and Gomori (16) with dogs. That with ninhydrin is of interest in supporting their (16) statement concerning the specificity of alloxan with respect to its ability to produce diabetes.

## SUMMARY

1. A gasometric, titrimetric, three colorimetric, and one fluorometric methods for the determination of alloxan have been outlined.
2. The chemical properties of alloxan are discussed in relation to methods for its quantitative determination and its stability in physiological liquids.
3. In the presence of cyanide and oxygen, dialuric acid gives rise to peroxide.
4. Blood plasma and urine of normal humans and dogs contain less than 0.02 mg. of alloxan per 100 cc.

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# GASOMETRIC DETERMINATION OF GLUTAMINE AMINO ACID CARBOXYL NITROGEN IN PLASMA AND TISSUE FILTRATES BY THE NINHYDRIN-CARBON DIOXIDE METHOD

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## *Reactions of Glutamine on Which Present Determination Is Based*

Glutamine, like other  $\alpha$ -amino acids, possesses the necessary specific configuration to react with ninhydrin with the evolution of  $\text{CO}_2$ , and its carboxyl ( $\alpha$ -amino<sup>1</sup>) nitrogen can therefore be measured by methods already published (1). Glutamine is also readily converted practically quantitatively, by heating in solution under conditions defined with regard to pH and time, to pyrrolidonecarboxylic acid; this latter compound does not react with ninhydrin to evolve  $\text{CO}_2$ . If a solution containing glutamine is analyzed for carboxyl nitrogen with and without being subjected to conditions that effect conversion of glutamine to pyrrolidonecarboxylic acid, the difference in carboxyl nitrogen between the treated and untreated samples will be a measure of the glutamine present in the solution.

To determine the glutamine carboxyl nitrogen in deproteinized picric acid filtrates of blood plasma and tissues, samples of filtrate are analyzed for carboxyl nitrogen by the manometric ninhydrin- $\text{CO}_2$  method (1) with and without heating for 90 minutes at  $100^\circ$  in 0.08 M phosphate buffer of pH 6.5. The difference in carboxyl nitrogen between the heated and unheated samples of filtrate, multiplied by a factor to correct for a small loss of glutamine carboxyl nitrogen in the unheated sample that occurs during the course of the procedure, gives the glutamine carboxyl nitrogen in the filtrate analyzed. A concentration of 1.0 mg. of glutamine carboxyl nitrogen per 100 cc. in plasma (about the usual plasma concentration) can be determined to within  $\pm 5$  per cent; glutamine in an aqueous solution of the same concentration can be determined to within  $\pm 2$  per cent.

The application of the ninhydrin- $\text{CO}_2$  method to the determination of

<sup>1</sup> The term "carboxyl nitrogen" is used to indicate values calculated as 1 gm. atom of nitrogen per mole of  $\text{CO}_2$  evolved by reaction of  $\alpha$ -amino acids with ninhydrin (1). In the paper by Hamilton and Van Slyke (2) the term " $\alpha$ -amino nitrogen" was used synonymously. "Carboxyl nitrogen," though unconventional, is less likely to be confused with the term "amino nitrogen," which is reserved for nitrogen determined by the nitrous acid procedure of Van Slyke (3).

glutamine in blood plasma filtrates has already been outlined in principle in a brief communication (4). Independently Neuberger and Sanger (5) applied the same principle to the determination of glutamine in potato extracts. Certain significant variables such as pH, temperature, nature and concentration of buffers employed for the change of glutamine to pyrrolidonecarboxylic acid, that must be taken into consideration in the quantitative determination of glutamine, were not considered in their paper.

The method presented in this paper can be carried out with reasonable rapidity, with a fairly high degree of specificity, and with less preparative procedure than most of the methods reviewed below. The method also yields the total carboxyl nitrogen of the free amino acids present in the filtrate analyzed. This value, determined on the picric acid filtrate after precipitation with neutral lead acetate, is lower than that obtained on the filtrate before treatment with lead acetate by the method of Hamilton and Van Slyke (2); the total carboxyl nitrogen of plasma picric acid filtrates is reduced by about 5 per cent and tissue filtrates by about 10 per cent by the lead precipitation. It appears that the precipitation with lead removed small amounts of amino acids other than glutamine.

*Interfering and Non-Interfering Substances*—Ammonia, glucose, pyruvic acid, pyrrolidonecarboxylic acid, and  $\alpha$ -amino acids do not interfere in the determination. Neither asparagine nor glutamic acid interferes: the conditions used for ring closure of glutamine change the carboxyl nitrogen of these compounds less than 0.1 per cent. Urea, because of its spontaneous evolution of CO<sub>2</sub> in hot solution, is a disturbing factor if present in relatively large amounts. In glutamine determinations in blood plasma, urea is therefore routinely removed by urease. In the analysis of tissues for glutamine it is unnecessary to remove urea, provided it is not present in abnormally great amounts. Glutathione and ascorbic acid in tissues, and ascorbic acid in plasma, will give rise to positive errors of considerable magnitude in the analytical procedure, but both are conveniently removed by a single precipitation at pH 6.5 with neutral lead acetate.

The conditions influencing the speed and completeness of the change of glutamine to pyrrolidonecarboxylic acid have been subjected to detailed study, and the influence of pH, temperature, the nature and concentration of buffer on its stability were systematically investigated. The most pertinent data with respect to the effect of these variable factors are included in this paper.

*Previous Procedures for Glutamine Determination*—The classical method for the determination of amide nitrogen originated with Sachsse (6) who measured the ammonia nitrogen liberated from protein-free filtrates of plant tissues on hydrolysis with 4 N sulfuric acid at 100° for 4 hours. The

amide nitrogen of both glutamine and asparagine is liberated quantitatively as ammonia under the condition of hydrolysis devised by Sachsse (6).<sup>2</sup>

Chibnall and Westall (9) noted that the amide group of glutamine was extensively hydrolyzed if the solution was heated at 100° for 3 hours at pH 8, whereas asparagine was scarcely affected by this treatment. They also noted that not only the  $\alpha$ -amino nitrogen but also 84 per cent of the amide nitrogen of glutamine was determinable by the Van Slyke nitrous acid procedure for analysis of  $\alpha$ -amino acids (3) when the reaction with nitrous acid was carried out for 10 minutes. As the amide group was hydrolyzed, a decrease in amino nitrogen determinable by nitrous acid occurred which paralleled the ammonia production. Chibnall and Westall tentatively suggested that these changes occurred as a result of the conversion of glutamine to ammonia and glutamic acid, part of the glutamic acid being subsequently converted to pyrrolidonecarboxylic acid. They also suggested that the change of amino nitrogen could be taken as a measure of the glutamine in plant filtrates.

The work of Chibnall and Westall (9) was extended by Vickery, Pucher, Clark, Chibnall, and Westall (10) to provide a quantitative method for the determination of glutamine in plant tissue extracts by measurement of the ammonia liberated in 2 hours at 100° at pH 6.5. These authors were cognizant of the fact, experimentally investigated by Chibnall and Westall (9), that urea was an interfering substance, but considered the traces of urea likely to be encountered in plant tissue extracts to give rise to negligible errors. These authors also provided evidence that glutamine was converted to ammonia and pyrrolidonecarboxylic acid, and not to ammonia and glutamic acid as originally postulated by Chibnall and Westall (9).

Pucher and Vickery (11) have described a more specific method for the determination of glutamine, based on the work of Chibnall and Westall (9) and Vickery *et al.* (10), in which plant filtrates are heated under specific conditions to give practically quantitative conversion of glutamine to pyrrolidonecarboxylic acid. This substance was then extracted from the filtrate with ethyl acetate in a continuous extraction apparatus. Amino nitrogen determinations by the nitrous acid method (3) were carried out on aliquot portions of an aqueous solution of the extracted material before and after heating for 2 hours at 100° in the presence of 2 N HCl. The hydrolysis reconverted the pyrrolidonecarboxylic acid to glutamic acid and the increase in amino nitrogen was taken as a measure of the glutamine. The extraction technique of Pucher and Vickery (11) makes removal of ammonia and urea unnecessary, but the analytical procedure is somewhat lengthy.

<sup>2</sup> Although the classical method for the determination of amide nitrogen was devised by Sachsse (6), glutamine was discovered by Schulze (7) and asparagine was discovered by Vauquelin and Robiquet (8).



Krebs (12) found that hydrolysis of the amide nitrogen of glutamine was complete in 5 minutes at 100° in 5 per cent sulfuric acid, while 23.2 per cent of the amide nitrogen of asparagine was hydrolyzed under the same conditions. Urea was claimed not to be hydrolyzed by the procedure. Chibnall and Westall (9) found that 22.9 per cent of the nitrogen of urea was liberated as ammonia in 1 hour at 100° in 1 N sulfuric acid. Harris (13) recognized the importance of this fact in his investigation of the rate of hydrolysis of glutamine amide nitrogen in trichloroacetic acid filtrates of blood plasma and in spinal fluid and in 0.7 N hydrochloric acid. Harris measured the ammonia nitrogen in deproteinized filtrates incubated for 1 hour and 15 minutes at 70° in 10 per cent trichloroacetic acid; complete hydrolysis of glutamine amide nitrogen occurs under these conditions. Any ammonia nitrogen that was liberated in a second period of 1 hour and 15 minutes at 70° was considered to come from urea and was accordingly subtracted from the ammonia nitrogen produced during the first incubation period. Under these conditions only 12 to 15 per cent of asparagine amide nitrogen is liberated; no evidence of the presence of asparagine in blood plasma was obtained.

More recently Archibald (14) has developed a highly specific method in which the amide group of glutamine is hydrolyzed by the enzyme glutaminase. If a good source of enzyme is available (dog kidney), the precision and specificity of the method are undeniable.

### Apparatus

The *Van Slyke-Neill manometric apparatus*, *storage vessel for CO<sub>2</sub>-free 0.5 N NaOH*, *alundum pieces* to promote smooth boiling, *calibrated glass spoons* for measuring 100 mg. of ninhydrin, *water bath* for reaction vessels, and *wire basket* for holding reaction vessels are all as described for the manometric ninhydrin-CO<sub>2</sub> method of Van Slyke, Dillon, MacFadyen, and Hamilton (1) or for the manometric carbon determination of Van Slyke and Folch (15). The *all-glass reaction vessel*, *high temperature lubricant*, and *rubber connecting tubes* are as described for the manometric ninhydrin-CO<sub>2</sub> method for the determination of carboxyl nitrogen in blood filtrates by Hamilton and Van Slyke (2). For analysis of tissues an ordinary household *meat mincer* and a *Waring blender*, or other rapid mincer, are necessary.

### Reagents

*Ninhydrin*, approximately 0.5 N NaOH of minimal CO<sub>2</sub> content in nearly saturated NaCl solution, approximately 5 N NaOH, approximately 2 N lactic acid in nearly saturated NaCl solution, and 1 per cent picric acid (0.0437 N) are as described by Hamilton and Van Slyke (2). The following reagents are also needed.

*2.10 M phosphate buffer solution to give pH 6.7 when diluted.* 16.28 gm. of anhydrous  $\text{KH}_2\text{PO}_4$  and 12.92 gm. of anhydrous  $\text{Na}_2\text{HPO}_4$  are placed in a 100 cc. volumetric flask, dissolved in distilled water, and made to volume. To 25 cc. of water, 1 cc. of the buffer is added and the pH of the solution checked at the glass electrode. It should be  $\text{pH } 6.7 \pm 0.1$ .

*3 M acetate buffer solution to give pH 4.7 when diluted.* 17.2 cc. of glacial acetic acid and 50 cc. of 3 N NaOH are mixed and made to 100 cc. volume with water. When 0.1 cc. of this buffer is added to 5 cc. of water, the pH should be  $4.7 \pm 0.1$ . It is checked at the glass electrode.

*20 per cent neutral lead acetate solution.* 23.5 gm. of neutral lead acetate,  $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$  (equivalent to 20 gm. of  $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ ), are dissolved in water and made to 100 cc. volume. The solution is stored in a well stoppered bottle.

*0.2 N potassium iodate solution.* 0.714 gm. of potassium iodate is made to 100 cc. with water.

*Approximately 4.5 N acetic acid.* 25 cc. of glacial acetic acid are made up to 100 cc. with water.

*Urease of low amino acid content*, prepared according to the directions of Archibald and Hamilton (16).

*Approximately 0.75 N NaOH.*

*0.4 per cent brom-thymol blue indicator, aqueous* (17).

*0.4 per cent brom-cresol purple indicator, aqueous* (17).

*Approximately 1 per cent picric acid solution containing 3 per cent acetic acid.* 30 cc. of glacial acetic acid are diluted to 1 liter with 1 per cent picric acid solution.

## PROCEDURES

### *Determination of Glutamine in Plasma*

*Preparation of Plasma Filtrates*—For plasma the following procedure is applicable. Blood is drawn (15 cc.) and placed in a flask containing 1.5 mg. of heparin as anticoagulant. The blood is centrifuged at once and the plasma separated. For duplicate analyses, 5 cc. of plasma are placed in a 30 cc. volumetric flask, 10 mg. of urease of low amino acid content (16) are added, and the flask contents adjusted to between pH 6.5 and 7.0 by the addition of 1 N acetic acid, with a drop of 0.4 per cent brom-thymol blue as an internal indicator. The flask is then placed in a water bath at  $37^\circ$  for 60 minutes. No measurable loss of glutamine occurs, provided acetate, and not phosphate, is used to buffer the plasma. If the plasma becomes too alkaline, as indicated by the development of a deep blue color, 1 N acetic acid is added till the color is restored to green. If the pH were permitted to exceed 7, action of the urease would be retarded and decomposition of glutamine would become significant.

Plasma and urease proteins are precipitated by making the volume to 30 cc. with 1 per cent picric acid solution. If the plasma contains 100 mg. or more of urea nitrogen per 100 cc., the solution is made to 30 cc. by addition of the 1 per cent picric acid solution containing 3 per cent acetic acid. (The acetic acid is added in order to neutralize ammonia from the unusual amount of urea and to render the solution sufficiently acid to effect complete precipitation of the proteins by the picric acid.) After standing 10 minutes the solution is transferred to a 50 cc. round bottomed centrifuge tube and the precipitated proteins are separated by centrifugation for 5 minutes at 3000 R.P.M. The supernatant solution is filtered through a funnel containing a plug of cotton about the size of a bean inserted into the lower end of the funnel stem (a larger plug or a filter paper might absorb too much filtrate to leave enough for duplicate analyses).

*Removal of Ascorbic Acid with Neutral Lead Acetate*—A 25 cc. portion of the picric acid filtrate is pipetted into a 50 cc. Erlenmeyer flask and is followed by 1.0 cc. of 20 per cent neutral lead acetate solution. To the flask is added, with swirling, 1.0 cc. of 0.75 N NaOH solution; the flask is stoppered and shaken, and let stand 5 minutes. The precipitate is removed by pouring into a dry filter paper (Schleicher and Schüll, 7 cm.; a larger paper absorbs too much to obtain the 24 cc. of filtrate needed for the next precipitation). In a 25 cc. volumetric flask is placed 1.0 cc. of 2.10 M phosphate buffer, pH 6.7, and the flask is filled to the mark with the filtrate from the lead precipitation. This serves to adjust the solution to approximately pH 6.5 or a little higher, and practically all excess lead is precipitated as the insoluble phosphate. The precipitated lead phosphate is removed by filtration through a dry filter paper. A 5 cc. aliquot portion of this final filtrate is pipetted into each of four all-glass reaction vessels for the carboxyl nitrogen determination, and to each is then added 1 drop of 0.4 per cent brom-cresol purple indicator, a drop of octyl alcohol, and three alundum beads.

*Determination of Carboxyl Nitrogen, Including the  $\alpha$ -Nitrogen of Glutamine*—The contents of one pair of reaction vessels are then adjusted to a faint red-brown color (pH approximately 4.7) with 4.5 N acetic acid (2 to 3 drops) added dropwise. The reaction is then brought to pH  $4.7 \pm 0.1$ , by adding 0.1 cc. of 3 M acetate buffer, pH 4.7. These two vessels are then heated over the free flame of a micro burner for *exactly* 1.5 minutes to boil off CO<sub>2</sub> that is present, or that may be formed from substances unstable at 100° and pH 4.7. The reaction vessel is then cooled *immediately* in ice water for 2 minutes, and a 2nd drop of octyl alcohol is added. Minimal heating time is used in order to minimize conversion of glutamine to pyrrolidonecarboxylic acid.

As soon as the 2 minute cooling period is ended, the short rubber connecting tube is slipped over the side arm, 200 mg. (two 100 mg. spoonfuls)

of ninhydrin are dropped into the cold solution, the greased stopper is set in place, and the vessel is evacuated 30 seconds at a good water pump. The stopper is closed and the rubber connecting tube on the side arm closed with a glass plug, according to the technique described on p. 238 of the paper by Hamilton and Van Slyke (2). The vessels are immersed completely in the 100° bath for exactly 4 minutes to complete the reaction of  $\alpha$ -amino acids with ninhydrin.

*Determination of Carboxyl Nitrogen Minus That of Glutamine*—The second pair of vessels, the solutions in which have also been adjusted to pH 6.5, is evacuated at the water pump till the pressure is reduced below 25 mm., the stopper of each is closed, and the connecting rubber tube on the side arm plugged. The vessels are then immersed in the 100° bath for 90 minutes to convert glutamine to pyrrolidonecarboxylic acid. They are then removed, cooled, and opened, and their contents are adjusted to pH 4.7 as described for the preceding pair of tubes. The preformed CO<sub>2</sub> is removed, 200 mg. of ninhydrin are added, and the reaction vessels immersed for 4 minutes in the 100° bath, all as directed for the first two reaction vessels.

*Determination of CO<sub>2</sub>*—The CO<sub>2</sub> evolved in each vessel by the reaction of ninhydrin with  $\alpha$ -amino acids is measured manometrically as described on pp. 238-239 of the paper by Hamilton and Van Slyke (2).

*Determination of the c Correction*—For these determinations a simulated plasma filtrate is prepared by adding 11 cc. of 0.10 N NaOH to 50 cc. of 1 per cent picric acid and the volume is then made to 100 cc. with water; the pH of the solution is  $2.0 \pm 0.1$ . Portions of this simulated filtrate are treated with neutral lead acetate and phosphate buffer, exactly as for true plasma filtrates, to give a "blank" filtrate.

The *c* correction is the  $p_1 - p_2$  value obtained by analysis of the simulated plasma filtrate. The greater part of the *c* correction, 20 to 25 mm. of CO<sub>2</sub> pressure measured at 0.5 cc. volume, is due to carbonate in the 0.5 N NaOH used as a reagent in the carboxyl nitrogen determination, but 2 to 5 additional mm. are added by the other reagents.<sup>3</sup> The *c* correction is the same for unheated and heated samples of both plasma and tissue filtrates.

*Calculations*—The pressure,  $P_{CO_2}$ , of CO<sub>2</sub> from the amino acid carboxyl groups for both unheated and heated samples is calculated as:

$$P_{CO_2} = p_1 - p_2 - c$$

The carboxyl nitrogen is calculated as:

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$$\text{Mg. carboxyl nitrogen per 100 cc. plasma} = P_{CO_2} \times \text{factor} \times V$$


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<sup>3</sup> Some lots of ninhydrin give no blank; others, for reasons not clearly understood, do give a small blank value. A blank value from ninhydrin is also more pronounced in picrate-phosphate solution at pH 4.7 than for other conditions of analysis previously described (1).

The values for the factors are given in Column 1, Table I.  $P_{CO_2}$ , when multiplied by these factors, gives mg. of carboxyl nitrogen per sample analyzed.  $V$  is the cc. of filtrate analyzed equivalent to 100 cc. of plasma and converts mg. of carboxyl nitrogen per sample analyzed to mg. of carboxyl nitrogen per 100 cc. of plasma. For plasma  $V = 135.0$ , when the preparation of plasma filtrates is as described. For ease in calculating results the value of  $factor \times V$  is given in Column 2, Table I.

If the mg. of carboxyl nitrogen per 100 cc. of plasma determined in the unheated filtrate (glutamine carboxyl N included) are designated as  $A_0$ , and mg. of carboxyl nitrogen per 100 cc. of plasma for the filtrates heated 90 minutes (glutamine carboxyl N removed) are designated as  $A_{90}$ , the mg. of glutamine carboxyl nitrogen are calculated as

$$Mg. \text{ glutamine carboxyl nitrogen per } 100 \text{ cc. plasma} = (A_0 - A_{90}) \times 1.095$$

The factor 1.095 is an experimentally determined correction factor based on analysis of pure glutamine under identical analytical conditions. The determination of this factor is discussed in a later part of this paper.

#### *Determination of Glutamine in Tissues*

*Handling of Tissues*—Organ tissues are obtained from a freshly killed animal and are either worked up at once or are rapidly frozen by packing them in solid CO<sub>2</sub>. They are stored in a box cooled below  $-40^\circ$  with solid CO<sub>2</sub> until analyzed. Tissues have been stored packed in solid CO<sub>2</sub> for 2 to 3 months without change in glutamine content.

*Preparation of Filtrate*—The tissue to be analyzed is stripped of visible fat and connective tissue and passed twice through an ordinary meat mincer. It is unnecessary to decompose urea as in plasma, provided the urea is not elevated above normal values. A 20.0 gm. portion of the minced tissue is placed in a Waring blender charged with 10 cc. of 1 per cent picric acid for each gm. of tissue other than brain. A more dilute filtrate of brain tissue is desirable, and 15 cc. of 1 per cent picric acid per gm. of tissue are used. It has been found that, if a more concentrated filtrate of brain tissue is used, loss of glutamine occurs when the filtrate is treated with lead acetate in the next step of the analysis.

5 minutes "blending" with the picric acid solution suffice to mince all of the lumps of tissue and to precipitate the proteins.

Each tissue suspension is divided equally between two 250 cc. centrifuge cups and is centrifuged for 10 minutes at 2000 R.P.M. The centrifugate is filtered through dry filter paper. Most organ tissues treated in this way give clear filtrates, but brain, liver, and gastrointestinal tract give cloudy filtrates. This cloudiness is removed by the next step in the procedure.

*Removal of Glutathione and Ascorbic Acid with Neutral Lead Acetate*—A 20 cc. portion of the picric acid filtrate is pipetted into a 25 cc. volumetric

TABLE I

*Factors by Which  $P_{CO_2}$  Is Multiplied to Obtain Mg. of Carboxyl Nitrogen per Sample of Filtrate Analyzed, and Mg. of Carboxyl Nitrogen per 100 Cc. of Plasma, per 100 Gm. of Tissue (Except Brain Tissue), and per 100 Gm. of Brain Tissue*

The 5 cc. portion of filtrate analyzed represents 0.724 cc. of plasma, 0.291 gm. of tissue (except brain), and 0.200 gm. of brain tissue.

$S = 3.00$  and  $\alpha = 0.5$  ( $i = 1.006$ ) in all analyses, with reagents made up in 25 per cent NaCl solution as described by MacFadyen (18).

Temperature	Sample analyzed (1)	Plasma (2)	Tissue (except brain) (3)	Brain (4)
°C.				
15	0.0004020	0.0543	0.1383	0.2010
16	04	41	77	02
17	0.0003987	38	72	0.1994
18	70	36	66	85
19	54	34	60	77
20	38	32	55	69
21	22	29	49	61
22	06	27	44	53
23	0.0003890	25	38	45
24	75	23	33	38
25	61	21	28	30
26	46	19	23	23
27	31	17	18	16
28	16	15	13	08
29	02	13	08	01
30	0.0003787	11	03	0.1894
31	72	09	0.1298	86
32	58	07	93	79
33	45	06	88	72
34	31	04	83	66
35	17	02	79	58

If excessive amounts of carboxyl nitrogen are encountered which necessitate measuring the evolved  $CO_2$  at 2.00 cc. volume, the factors in the column under  $\alpha = 2.000$  in Table I of the paper by MacFadyen are used to multiply  $P_{CO_2}$  to obtain mg. of carboxyl nitrogen per sample analyzed. The mg. of carboxyl nitrogen per sample analyzed, multiplied by 135.0, gives mg. of carboxyl nitrogen per 100 cc. plasma, by 344, gives mg. of carboxyl nitrogen per 100 gm. of tissue (except brain), and by 500, gives mg. of carboxyl nitrogen per 100 gm. of brain.

flask, followed by 1 cc. of 20 per cent neutral lead acetate solution. Then 1.0 cc. of 0.75 N NaOH is added, a drop at a time with mixing of the flask contents after each addition. The mixture is made to volume with water,

let stand 10 minutes, and is then transferred to a centrifuge tube, and the precipitate is either centrifuged or filtered on a dry filter paper. To a 20 cc. portion of this filtrate, in a 25 cc. volumetric flask, is added 1 cc. of the 2.10 M phosphate buffer solution of pH 6.7, and the solution is made to volume with water. The precipitated lead phosphate is thrown down by centrifugation and the centrifugate filtered through a dry filter paper.

*Determination of Glutamine and Total Carboxyl Nitrogen*—In each of four all-glass reaction vessels for carboxyl nitrogen are placed 5 cc. of the final filtrate and to each is added 0.1 cc. of 0.2 N potassium iodate solution. This serves to convert any trace of glutathione that may have escaped precipitation by the lead to the oxidized (disulfide) form in which its carboxyl nitrogen is much more stable during the subsequent heating at 100°. The  $A_0$  and  $A_{90}$  carboxyl nitrogen values are determined as for plasma.

*Calculations*—As in analysis of plasma filtrates, the  $P_{CO_2}$  of CO<sub>2</sub> from the amino acid carboxyl groups of the unheated and heated samples is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

The determination of the  $c$  correction is the same as that for plasma. The carboxyl nitrogen is calculated as

$$\text{Mg. carboxyl nitrogen per 100 gm. tissue} = P_{CO_2} \times \text{factor} \times V$$

The values for the factors are given in Column 1, Table I.  $P_{CO_2}$ , when multiplied by these factors, gives mg. of carboxyl nitrogen per sample of filtrate analyzed.  $V$  is the cc. of filtrate analyzed equivalent to 100 gm. of tissue. If brain tissue is analyzed,  $V = 500$ ; for all other tissues  $V = 344$ . In calculating the value of  $V$ , it is assumed for practical purposes that 1 gm. of tissue occupies 1 cc. volume. For ease in calculating results, the values of  $\text{factor} \times V$ , for tissue (except brain) and for brain tissue, are given in Columns 3 and 4, Table I.

The glutamine carboxyl nitrogen content of the tissue analyzed is calculated as

$$\text{Mg. glutamine carboxyl nitrogen per 100 gm. tissue} = (A_0 - A_{90}) \times 1.095$$

The factor 1.095 corrects for losses of glutamine carboxyl nitrogen that occur under the conditions of analysis. Its determination is discussed later in the paper.

No investigation has been carried out on tissues of animals with other than normal urea content.

*Determination of Glutamine in Whole Blood and Red Blood Cells*—The determination of glutamine in whole blood or red blood cells by the methods described for tissues is impracticable because of the 5- to 6-fold dilution that occurs during the removal of blood proteins and glutathione. The

difference in carboxyl nitrogen between the  $A_0$  and  $A_{90}$  analyses is too small to measure with a sufficient degree of accuracy. A few trial analyses indicate that the glutamine concentration per 100 cc. of red blood cells (human) is about the same as the value per 100 cc. of plasma from the same blood sample.

#### EXPERIMENTAL

*Reaction of Glutamine<sup>4</sup> with Ninhydrin*—Because of the rapidity with which glutamine undergoes ring closure by the elimination of ammonia to form pyrrolidonecarboxylic acid, especially at 100° in aqueous solution, the yield of carboxyl nitrogen obtained by heating with ninhydrin is always less than the calculated theoretical yield of 1 gm. atom per mole; a small proportion of the glutamine changes to pyrrolidonecarboxylic acid before there is time for complete reaction with ninhydrin, and is thus prevented from reacting with ninhydrin. In acetate-buffered solutions of pH 4.7, with acetate concentrations 0.01, 0.1, and 0.5 M respectively, the yield of carboxyl nitrogen obtained from glutamine heated with ninhydrin was 0.995, 0.995, and 0.983 gm. atoms respectively per mole of glutamine. In phosphate solutions of the same molar concentration and pH, the yield of carboxyl nitrogen was 0.995, 0.983, and 0.962 gm. atoms per mole respectively. In 0.1 M citrate or lactate of pH 4.7 the yield was 0.983 gm. atom per mole. The yield of carboxyl nitrogen in buffered solutions was not altered by the addition of picrate ion in the concentration encountered in plasma and tissue filtrates. The effects of acetate, phosphate, citrate, and lactate solutions on the speed of ring closure of glutamine are discussed in later sections of this paper.

If the ninhydrin reaction is carried out in solutions more acid than pH 4.7, the yield of carboxyl nitrogen is reduced below the values noted above because of more rapid ring closure of glutamine; larger fractions of the glutamine undergo ring closure before reaction with ninhydrin occurs. For example, at pH 2.0 in 0.5 per cent picric acid solution the carboxyl nitrogen yield is 0.962 gm. atom per mole of glutamine, as compared with 0.995 gm. atom in 0.5 per cent picric acid plus NaOH and acetate buffer to bring the pH to 4.7.

The preliminary heating over the free flame of a micro burner for 1.5 minutes to remove preformed  $\text{CO}_2$  causes sufficient conversion of glutamine to pyrrolidonecarboxylic acid to decrease measurably the glutamine carboxyl nitrogen, the magnitude of the decrease depending on the nature and concentration of buffer in the solution. Under the conditions outlined for

<sup>4</sup> The gift of a generous sample of glutamine from Dr. G. W. Pucher and Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station, New Haven, is gratefully acknowledged.



analysis of plasma and tissue filtrates, the loss is 5 per cent if the solution is heated 1.5 minutes to remove preformed CO<sub>2</sub> and cooled immediately thereafter in ice water.

For any given set of conditions, consecutive analytical values for carboxyl nitrogen usually differ by less than  $\pm 1$  per cent. The following experiments are illustrative.

A 10 mg. sample of glutamine was dissolved in 100 cc. of an aqueous solution of 0.5 per cent picric acid (previously adjusted to pH 2.0 by the addition of NaOH to simulate plasma filtrates with respect to picric acid concentration and pH) and 5 cc. portions of this solution were placed in each of six all-glass reaction vessels. The contents of the vessels were adjusted to pH 4.7, and then phosphate was added to give the same concentration as was found in the A<sub>0</sub> analyses described for the determination of glutamine in picric acid filtrates. Preformed CO<sub>2</sub> was removed by evacuation of the vessels at the water pump, 200 mg. of ninhydrin were added, and the vessels were placed in the 100° bath for exactly 4 minutes. The analytical values obtained were 0.990, 0.974, 0.986, 0.981, 0.984, and 0.987, average 0.984, gm. atoms of carboxyl nitrogen per mole of glutamine. In two other series of analyses, preformed CO<sub>2</sub> was removed by 1.5 minutes heating over the free flame of a micro burner. The analytical values were 0.929, 0.939, 0.937, 0.937, 0.938, 0.921 gm. atoms of carboxyl nitrogen per mole of glutamine and 0.909, 0.931, 0.916, 0.939, 0.931, 0.932 gm. atoms per mole respectively, giving an average yield of 0.930 gm. atom per mole for these twelve analyses.

*Conditions for Maximal Formation of Pyrrolidonecarboxylic Acid from Glutamine at pH 6.5*—Although the ammonia formed on ring closure of glutamine is 1 mole per mole, the maximal yield of pyrrolidonecarboxylic acid, as measured by the disappearance of carboxyl nitrogen, was found to be 0.99 mole per mole because 1 per cent of the product is glutamic acid and 99 per cent pyrrolidonecarboxylic acid. Under the conditions of the analysis, after all of the glutamine amide nitrogen is split off as ammonia, the equilibrium appears to be reached when pyrrolidonecarboxylic acid and glutamic acid are present in the ratio 100:1.

This was shown by the following experiment: 5 cc. portions of 0.01 per cent glutamine solution in 0.5 per cent picric acid, pH 2.0, were placed in five all-glass reaction vessels and adjusted to pH 6.5 with 0.105 M final concentration of phosphate buffer. The vessels were partially evacuated and immersed in the 100° bath for 90 minutes. After removing them from the bath, the contents were at once cooled and adjusted to pH 4.7 and the analysis was carried out exactly as for those samples in the previous experiment. The carboxyl nitrogen was 0.01, 0.01, 0.01, 0.01, and 0.01 gm. atoms per mole of original glutamine. Aliquot samples of the same solution heated in the 100° bath for 120 minutes gave no further decrease in carboxyl nitrogen, nor could the residual carboxyl nitrogen be reduced below 0.01 gm. atom per mole by effecting ring closure at other pH values, in other buffer concentrations, or in other buffer mixtures.

In buffered solutions of pH 6.5 of 0.08 M phosphate concentration, the residual carboxyl nitrogen is slightly but definitely higher: the average of forty-six separate determinations was 0.016 gm. atom per mole.

On heating glutamine at 100° in solutions of pH 3 in acetate or phosphate solutions of 0.008, 0.08, and 0.4 M concentration, the residual carboxyl nitrogen was 0.03 to 0.05 gm. atom per mole of original glutamine. At pH 6.5 the residual carboxyl nitrogen was 0.02 to 0.03 gm. atom per mole in acetate solutions of the same molar concentration and 0.01 to 0.02 gm. atom per mole in phosphate solutions. These results are in keeping with the findings of Wilson and Cannan (19), who showed that glutamic acid and pyrrolidonecarboxylic acid form a reversible system whose equilibrium constant is governed by the pH of the solution. At pH 6.5 the equilibrium yields nearly complete pyrrolidonecarboxylic acid formation. At pH 3

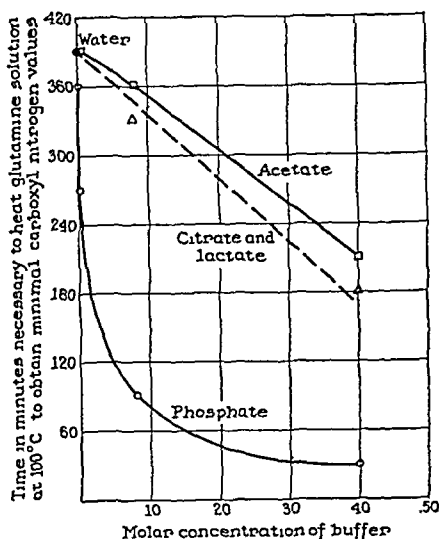


FIG. 1. Ring closure of glutamine at pH 6.5

the proportion of glutamic acid is increased and the proportion of pyrrolidonecarboxylic acid decreased.

*Conditions Which Influence Duration of Heating Glutamine Solutions at 100° Required to Give Minimal Carboxyl Nitrogen Values*—The time necessary to heat glutamine solutions to obtain maximal conversion to pyrrolidonecarboxylic acid depends on the choice of buffer selected, its concentration, the pH, and the temperature.

In Fig. 1 the time necessary to heat glutamine in aqueous solutions of pH 6.5 at 100° to give minimal carboxyl nitrogen values (*i.e.*, maximal formation of pyrrolidonecarboxylic acid) is plotted against molar concentrations of the solution. It is seen that at constant pH of 6.5 for all molar concentrations of phosphate investigated the time of heating necessary for

maximal formation of pyrrolidonecarboxylic acid is very much less than in solutions of lactate, citrate, or acetate of the same molar concentration and pH.

The curves in Fig. 1 were obtained by heating glutamine in solutions adjusted to pH 6.5 in the molar concentrations shown. Each curve was obtained by heating aliquot samples of glutamine in all-glass reaction vessels at 100° for 0.5, 1, 2, 3, 5, and 7 hours. After they were removed from the bath and cooled, a drop of brom-cresol purple indicator was added, followed by 1 N HCl, until the solution became a faint brown color (approximately pH 4.7). Acetate buffer (0.1 cc. of 3 M) was added to

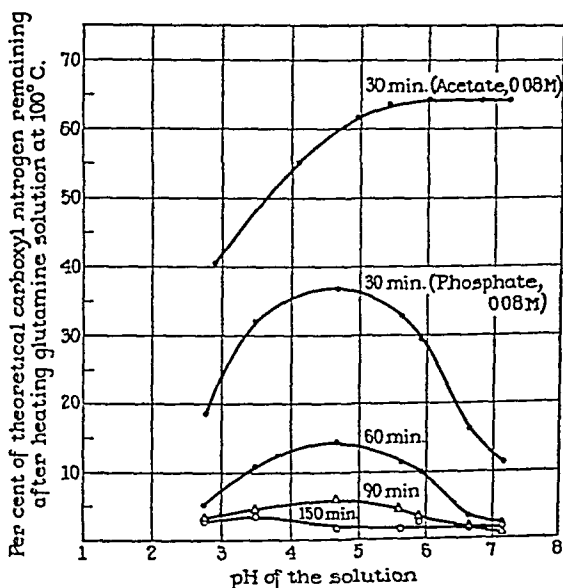


FIG. 2. Ring closure of glutamine heated at 0.08 M phosphate buffer at 100° for 30, 60, 90, and 150 minutes

bring the solution to pH  $4.7 \pm 0.1$ . Preformed CO<sub>2</sub> was removed by preliminary heating over the free flame of a micro burner for exactly 1.5 minutes. On cooling, 200 mg. of ninhydrin were added and the carboxyl nitrogen was determined. The residual carboxyl nitrogen, computed as a per cent of the carboxyl nitrogen initially present in the solution, was plotted against the time of heating and a smooth curve drawn through the points and the time necessary to heat at 100° to obtain minimal values for carboxyl nitrogen read from the curve.

In Fig. 2 the percentage of theoretical carboxyl nitrogen that remains after heating glutamine solutions for 30, 60, 90, and 150 minutes at 100° is plotted against the pH of the solution; the effect in 0.08 M phosphate is compared with that in 0.08 M acetate. Ring closure is slowest in the

phosphate solutions when the pH is 4.7 and is faster for values that are more acid or more alkaline than 4.7. In acetate solution, ring closure is slower than in phosphate solution, and the curve for 30 minutes heating, which is representative of the behavior in acetate, shows no acceleration of ring closure as the pH increases above 4.7.

The differences noted for the speed of ring closure of glutamine in acetate, citrate, or lactate solutions of pH 6.5, as compared with the speed of ring closure in phosphate solutions of the same pH and molar concentration, serve to emphasize that the behavior of glutamine when heated in aqueous solution is dependent not only upon the time, temperature, and hydrogen

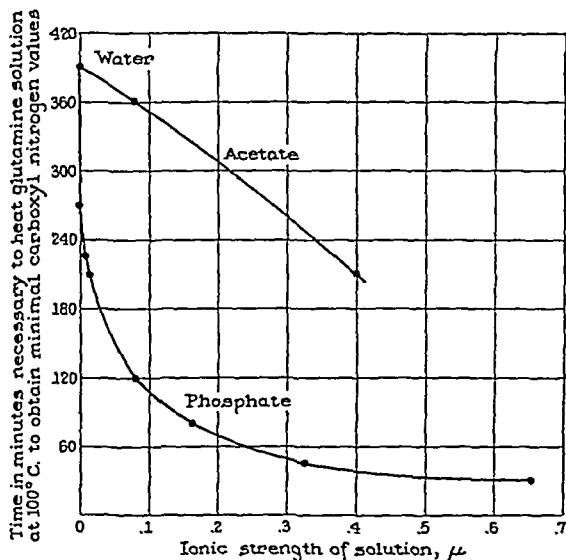


FIG. 3. Ring closure of glutamine at pH 6.5

ion concentration but also the concentration and kind of buffer constituents of the solution. For example, to change from one buffer system without phosphate to one containing phosphate will markedly alter the time necessary to heat glutamine in solution to obtain maximal ring closure.

Fig. 3 shows the data of Fig. 1, plotted against ionic strength of the solutions  $\mu$ , as abscissae. It is seen that the difference in speed of ring closure is also independent of the ionic strength of the solutions. The results indicate that, at least at pH 6.5, ring closure is more rapid in the presence of phosphate than of acetate, citrate, or lactate buffers, when they are in either the same molar concentration or the same ionic strength as the phosphate.

*Effect of Temperature on Speed of Formation of Pyrrolidonecarboxylic Acid from Glutamine*—Glutamine in solutions of 0.5 per cent picric acid, pH 2, loses 8 to 10 per cent of its carboxyl nitrogen per 24 hours when kept at 25°. When similar solutions are stored at 4°, the loss of carboxyl nitrogen is about 0.5 to 0.8 per cent per 24 hours. When working with plasma or tissue filtrates containing glutamine, it is therefore desirable to analyze them for carboxyl nitrogen at once.

*Determination of Empirical Correction Factor for Glutamine Analysis*—The yield of glutamine carboxyl nitrogen has been shown to be 0.930 gm. atom per mole of glutamine when preformed CO<sub>2</sub> is removed by 1.5 minutes heating over the free flame of a micro burner, and the reaction with ninhydrin is carried out at pH 4.7 in 0.08 M phosphate concentration. The residual carboxyl nitrogen, after glutamine is heated in solution of pH 6.5 and 0.08 M phosphate concentration for 90 minutes at 100°, has been shown to be 0.016 gm. atom per mole of glutamine. The difference,  $0.930 - 0.016 = 0.914$  gm. atom, is the loss of carboxyl nitrogen equivalent to 1 mole of glutamine when solutions are analyzed before and after heating under these conditions. To correct for losses of glutamine carboxyl nitrogen when determined by the described procedure, the analytical values are accordingly multiplied by  $1/0.914$  or 1.095.

*Non-Interference by Glutamic Acid and Asparagine*—Not only glutamine but also glutamic acid undergoes anhydride formation with conversion to pyrrolidonecarboxylic acid. However, the rate of ring formation from glutamic acid is so slow under the conditions used in the analysis of glutamine that less than 0.1 per cent of the glutamic acid is converted during the 90 minute heating period.

Asparagine, if present, will introduce no significant error because its acid amide group is hydrolyzed to less than 0.1 per cent under the conditions of the analysis.

*Non-Interference of Other Components of Plasma and Tissue Filtrates, Other Than Glutathione, Ascorbic Acid, or Urea*—Plasma and tissue filtrates, free of urea, glutathione, and ascorbic acid, were hydrolyzed for 5 minutes with 2 N hydrochloric acid at 100° to convert all glutamine to ammonia and glutamic acid. Excess hydrochloric acid was removed by vacuum distillation, and the dry residue taken up in distilled water and made to the original volume. 5 cc. aliquot portions of this solution were analyzed for carboxyl nitrogen before and after heating for 90 minutes as described for plasma filtrates. The carboxyl nitrogen values for unheated and heated samples of plasma filtrate prepared in this manner were 5.98 and 5.96 mg. per 100 cc. respectively. A heart muscle filtrate, also prepared similarly, with the exception that urea was not removed, gave 26.25 and 26.18 mg. of carboxyl nitrogen per 100 gm. of tissue for the unheated and heated samples

respectively; the small difference noted here may be attributable to the presence of the urea in the filtrate.

The addition of glucose or pyruvic acid has similarly no demonstrable effect on the carboxyl nitrogen values of the unheated and heated samples.

Very labile non-specific sources of  $\text{CO}_2$  such as acetoacetic acid, bicarbonate- $\text{CO}_2$ , and  $\text{CO}_2$  in solution, are eliminated by the preliminary heating for 1.5 minutes over the free flame of a micro burner, before reaction with ninhydrin is carried out (1).

*Interference of Glutathione, Ascorbic Acid, and Urea*—Since glutamic acid is linked through its  $\delta$ -carboxyl group to the  $\alpha$ -amino group of cysteine in glutathione, the  $-\text{CH}(\text{NH}_2)\cdot\text{COOH}$  group of the glutamic acid is free and reacts on heating in aqueous solution with ninhydrin with the evolution of  $\text{CO}_2$  equivalent to 1 atom of carboxyl nitrogen per mole of the peptide (1).

It is known that glutathione breaks down spontaneously in aqueous solution at neutral pH with the liberation of its glutamic acid moiety as pyrrolidonecarboxylic acid. Heating reduced glutathione in 0.08 M phosphate buffer at pH 6.5 for 90 minutes at  $100^\circ$  causes a loss of 47 per cent of its carboxyl nitrogen, presumably due to the formation of pyrrolidonecarboxylic acid. On the other hand, heating oxidized glutathione with the SH— group in the disulfide  $-\text{S}-\text{S}-$  form causes a loss of only 8 per cent of its carboxyl nitrogen under similar conditions. The explanation of this finding is being further investigated.

It was found that 90 per cent of reduced glutathione, in the concentrations encountered in tissue filtrates, is precipitated from deproteinized picric acid filtrates at pH 6.5 by neutral lead acetate. It was also found that potassium iodate rapidly oxidizes glutathione in picric acid filtrates, at room temperature, and the presence of potassium iodate does not interfere with the subsequent reaction of amino acids with ninhydrin. Accordingly, when most of the glutathione is removed from tissue filtrates by precipitation with neutral lead acetate and the glutathione remaining after the precipitation with lead acetate is oxidized by the addition of 0.2 N potassium iodate, glutathione is eliminated as a source of significant analytical error.

Since there is little or no glutathione in plasma (Dohan and Woodward (20)), potassium iodate is omitted in the analytical procedure for plasma filtrates.

Ascorbic acid reacts with ninhydrin and, under the conditions for the determination of carboxyl nitrogen in plasma and tissue filtrates, evolves approximately 0.5 mole of  $\text{CO}_2$  per mole of ascorbic acid. If ascorbic acid is heated at  $100^\circ$  for 90 minutes at pH 6.5 in 0.08 M phosphate and 0.5 per cent picric acid solution, and then submitted to the ninhydrin analysis, only 0.02 mole of  $\text{CO}_2$  is evolved per mole of ascorbic acid. Its presence

will therefore increase the apparent glutamine by 0.48 mole per mole of ascorbic acid. Ascorbic acid is almost quantitatively precipitated (96 to 98 per cent) from solution by neutral lead acetate at pH 6.5. The residual ascorbic acid gives rise to negligible errors in the analysis of the filtrates. The single treatment with lead acetate thus serves to remove both glutathione and ascorbic acid practically quantitatively from picric acid filtrates.

Urea, unless removed from plasma filtrates, introduces a small positive error in the carboxyl nitrogen determinations. As previously shown (1, 2), a small fraction of the urea is hydrolyzed with the evolution of CO<sub>2</sub> when the filtrate is heated at 100° during the course of reaction of amino acids with ninhydrin. This non-specific CO<sub>2</sub> is measured along with CO<sub>2</sub> evolved from reaction of amino acids with ninhydrin. In tissue analyses the effect of urea is so slight that it falls within the limits of error of the glutamine method. In plasma, however, the concentration of glutamine is much smaller, and consequently the ratio of urea to glutamine is higher than in tissues. The elimination of urea from plasma by preliminary treatment with urease is accordingly advisable.

*Determination of Glutamine Added to Plasma and Tissues*—In Table II is shown the determination by methods described in this paper of glutamine carboxyl nitrogen added to human and dog plasma and to dog tissues. In all cases the recovery of added glutamine is within  $\pm 1$  per cent. Table II also serves to indicate glutamine carboxyl nitrogen values encountered in human and dog plasmas (Column 6). The glutamine carboxyl nitrogen values of the various tissues (Column 6) are also representative of these tissues. A more detailed comparison of glutamine values in plasma and tissues is reserved for another paper (21).

#### DISCUSSION

Examination of the data in Table I of the paper by Vickery *et al.* (10) reveals that for solutions of pH 5.0, 5.3, 5.5, and 5.7 the per cent of glutamine remaining in solution after 2 and 3 hours at 100° plotted logarithmically against time of heating the solutions yields points that lie on straight lines, indicating that the conversion of glutamine to pyrrolidonecarboxylic acid follows the pattern of a monomolecular reaction. Extrapolation to the time necessary to heat glutamine at 100° to get 98 to 99 per cent conversion shows that at pH 5.0, 5.3, 5.5, and 5.7 it would take 6 hours or longer. At these pH values a succinic acid-borax buffer was employed. At pH 6.0 and higher, when a dihydrogen phosphate-borax buffer was used, the time necessary to heat at 100° to get 98 to 99 per cent ring closure was 2 hours at most, thereby demonstrating that at pH 6.0 ring closure was 3 times as fast as at pH 5.7. It seems probable that the change from a non-phosphate to a phosphate buffer, rather than the 0.3 change in pH, was

responsible for the greater part of this 3-fold increase in the rate of ring closure.

TABLE II

*Determination of Glutamine Carboxyl Nitrogen Added to Human and Dog Plasma and Dog Tissues*

Glutamine added to	Plasma or tissue + glutamine			Plasma or tissue			Increase in glutamine carboxyl N found	Glutamine carboxyl N added	Per cent recovery
	Total carboxyl N		Glutamine carboxyl N	Total carboxyl N		Glutamine carboxyl N*			
	A <sub>0</sub>	A <sub>100</sub>		A <sub>0</sub>	A <sub>100</sub>				
	(1)	(2)	$A_0 - A_{100} \times \frac{1.095}{((1) - (2))} \times \frac{1.095}{(3)}$	(4)	(5)	$A_0 - A_{100} \times \frac{1.095}{((4) - (5))} \times \frac{1.095}{(6)}$	(3) - (6)	(8)	$\frac{(7)}{(8)} \times 100$
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
Plasma 1, human, fasting.....	14.48	2.47	13.15	3.09	2.52	0.62	12.53	12.52	100.1
Plasma 2, human, lyophilized.....	17.01	5.08	13.06	5.44	4.80	0.70	12.36	12.41	99.6
Plasma 3, human, non-fasting.....	17.65	4.28	14.64	5.19	4.11	1.18	13.46	13.59	99.1
Plasma 4, human, non-fasting.....	18.38	4.86	14.65	5.64	4.61	1.13	13.51	13.56	99.6
Plasma 5, human, lyophilized.....	15.40	5.06	11.32	5.52	4.80	0.79	10.53	10.55	99.8
Plasma, dog, fasting.....	19.28	6.20	14.32	7.14	5.95	1.29	13.03	13.16	99.0
“ “	18.02	4.26	15.07	5.26	4.05	1.32	13.75	13.78	99.8
	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	mg. per 100 gm.	
Heart muscle, dog, normal.....	57.4	19.4	41.6	34.5	18.6	17.3	24.3	24.5	99.2
Kidney, dog, normal.....	54.3	17.7	40.1	15.1	14.3	0.9	39.2	39.6	99.0
Liver, dog, anoxic.	35.7	14.1	23.6	13.2	12.2	1.1	22.5	22.4	100.5
Brain, dog, normal†.....	57.9	23.3	34.6	25.0	21.0	4.0	33.5	33.5	100.0

\* Factor of 1.095 to correct for losses of glutamine carboxyl nitrogen; for explanation see the text.

† Brain proteins precipitated by 1 gm. of tissue plus 15 cc. of 1 per cent picric acid; 1 gm. of tissue plus 10 cc. of 1 per cent picric acid for all other tissues.

It is also of some interest to note that, though the isoelectric point of glutamine was found by Melville and Richardson (22) to be 5.65, the point of maximal stability of glutamine in phosphate solutions was found to be very close to pH 5.0, while in acetate solutions the stability was maximal at



pH 5.0 and undiminished at pH 7.0. This shows that other factors than proximity to the isoelectric point of glutamine are concerned with its stability.

Observations have also been made in the literature on the stability of glutamine in the preparation of bacteriological media for the growth of organisms that require glutamine as an essential nutrient factor. McIlwain (23) observed that one medium containing agar was able to withstand autoclaving at 120° for 20 minutes, while in peptone infusion not containing agar some destruction of glutamine occurred. In view of the rapid destruction of glutamine at neutral pH in the presence of low concentrations of phosphate as well as the markedly increased rate of destruction of glutamine with increasing concentrations of phosphate, reported in this paper, a possible explanation of these observations lies in the presence and concentration of phosphate rather than in the presence or absence of agar; difference in pH of the media (not given in McIlwain's paper (23)) could also account for the difference noted. The possible protecting action of agar has not been explored in this study, but plasma proteins have not been found to exert any protecting influence on the breakdown of glutamine.

The present method for the determination of glutamine distinguishes clearly between glutamine and its next lower homologue, asparagine; higher homologues are not available for investigation. Except for glutathione, which might be considered a  $\delta$ -N-substituted derivative of glutamine, no  $\delta$ -N-substituted derivatives of glutamine have been available for study. In consequence no prediction can be made as to whether the method will distinguish between glutamine and, for example,  $\delta$ -N-formyl glutamine which, as judged by the behavior of glutamine and glutathione, would probably exhibit the property of ring closure with consequent loss of carboxyl nitrogen.

It is a privilege to acknowledge the author's indebtedness to Dr. D. D. Van Slyke for constant help and encouragement through the entire course of this work. Constant collaboration with Dr. R. M. Archibald has been not only profitable but a great pleasure, and the author's indebtedness to him also is freely acknowledged.

#### SUMMARY

1. Glutamine is estimated from the decrease in carboxyl nitrogen caused by heating at 100° for 90 minutes at pH 6.5. The glutamine is changed to ammonia and pyrrolidonecarboxylic acid, with disappearance of the carboxyl nitrogen. The carboxyl nitrogen is determined by the ninhydrin-CO<sub>2</sub> method of Van Slyke, Dillon, MacFadyen, and Hamilton (1).

2. Ammonia, glucose, pyruvic acid, acetoacetic acid, pyrrolidonecarboxylic acid, asparagine, glutamic acid, or other free  $\alpha$ -amino acids do not interfere in the determination of glutamine. Glutathione, ascorbic acid, and urea do interfere in the analysis for glutamine, but the two former are removed by a single precipitation with neutral lead acetate at pH 6.5, while the latter is decomposed by the action of urease.

3. Glutamine carboxyl nitrogen in plasma and tissues can be determined to within 0.05 mg. of carboxyl nitrogen per 100 cc. of plasma or per 100 gm. of tissue (wet weight). The total carboxyl nitrogen of the picric acid filtrate after treatment with lead acetate is determined at the same time.

4. The effects of pH, temperature, type and molar concentration of buffer solution on the reaction of glutamine with ninhydrin have been investigated. Also the time necessary to heat glutamine in solutions of phosphate, citrate, lactate, and acetate at pH 6.5 at 100° to obtain maximal formation of pyrrolidonecarboxylic acid has been determined. Ring closure at pH 6.5 is faster in phosphate solutions than in citrate, acetate, or lactate solutions of the same molar concentration.

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# GLUTAMINE: A MAJOR CONSTITUENT OF FREE $\alpha$ -AMINO ACIDS IN ANIMAL TISSUES AND BLOOD PLASMA

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The present paper, one of a series from this laboratory on the chemistry (1-4) and physiology (5) of glutamine, reports evidence that glutamine or a glutamine-like substance is a major constituent, present in human and dog plasma and in all dog tissues studied so far. This substance occurs in the free form. In blood plasma glutamine constitutes 15 to 25 per cent of the free circulating  $\alpha$ -amino acids and preliminary results have already been briefly reported (1) and confirmed (3-6).

In dog heart muscle, glutamine constitutes approximately 60 per cent and in kidney tissue about 4 per cent of the free  $\alpha$ -amino acids. Glutamine values for other tissues examined are intermediate between these extremes. These results have confirmation by the independent work of Ferdman, Frenkel, and Silakova (7) published in Russia.

Methods for the quantitative determination of glutamine carboxyl nitrogen<sup>1</sup> in plasma and tissues by the ninhydrin- $\text{CO}_2$  method of Van Slyke and his associates (8-10) are published in the accompanying paper (4). In the present paper the ninhydrin- $\text{CO}_2$  method is used in conjunction with amino nitrogen determinations by the nitrous acid method of Van Slyke (11) and with determinations of ammonia liberated by acid hydrolysis, the ammonia being determined by the micro vacuum distillation technique of Archibald (12). The results provide evidence that the unstable "glutamine-like" (1) component of plasma and tissue filtrates is in fact glutamine.

Glutamine has been the subject of study for many years by plant biochemists, in recent years notably by Vickery, Pucher, Clark, Chibnall, and Westall (13), and the literature concerned with its metabolic rôle in plant biochemistry has been reviewed by Chibnall (14). It has also been found to be of importance in bacterial nutrition (15, 16). But the study of glutamine by biochemists has not been extensive, and the significant facts now known concerning its physiological rôle in animal biochemistry can

<sup>1</sup> The term "carboxyl nitrogen" is used to indicate values calculated as 1 gm. atom of nitrogen per mole of  $\text{CO}_2$  evolved by reaction of  $\alpha$ -amino acids with ninhydrin (8-10). The term amino nitrogen is reserved for nitrogen determinable by the nitrous acid procedure of Van Slyke (11).

be briefly summarized. Its physiological importance in plant, animal, and bacterial metabolism is thoroughly reviewed by Archibald (unpublished work).

Thierfelder and Sherwin (17) showed that phenylacetic acid, when fed to humans, was excreted in the urine and conjugated mainly with glutamine. Their findings indicated the presence and synthesis of glutamine in the body where it could function as a detoxifying agent. Power (18) showed that phenylacetic acid was also conjugated similarly in the chimpanzee. The interesting fact that this conjugation is apparently confined to man and the higher apes was pointed out by Young (19).

Krebs (20) demonstrated that glutamine can be synthesized *in vitro* from ammonia and glutamic acid by liver slices. Örström, Örström, Krebs, and Eggleton (21) demonstrated that glutamine could also be synthesized by liver slices from pyruvate and ammonia in the presence of a source of energy such as glucose. The product of enzymatic synthesis was proved by isolation and identification of the crystalline compound. Krebs (20) also obtained from liver, brain, retina, and kidney active preparations of an enzyme or enzymes which he called glutaminase and which split glutamine into ammonia and glutamic acid. Leuthardt and Glasson (22), from *in vitro* studies of tissue slices, suggested that glutamine can function as an ammonia donator and acceptor, and Bach (23), also on the basis of *in vitro* studies, postulated an amide-nitrogen cycle as a mechanism of ammonia transport in the body. No evidence that these mechanisms operate in the intact animal was given by these authors.

McIlwain, Fildes, Gladstone, and Knight (15) isolated glutamine as the crystalline compound from horse skeletal muscle during the course of work on essential nutrient factors in cultural media for hemolytic streptococci.

Ferdman, Frenkel, and Silakova (7) reported the isolation of glutamine from horse brain. They also determined the amount of ammonia liberated by 5 minutes hydrolysis with 2 N sulfuric acid at 100° in trichloroacetic acid filtrates of heart muscle, skeletal muscle, liver, and kidneys of horse, dog, cat, rabbit, marmot, pigeon, crayfish, dog blood, human urine, and cerebrospinal fluid; this labile ammonia was attributed to the amide nitrogen of glutamine. The amount of this labile nitrogen was higher in heart muscle than in any other organs analyzed in all animals investigated, except the pigeon, in which it was highest in brain.

The presence of free glutamine or glutamine-like substance in blood plasma of dog and human, reported in a preliminary communication (1), has been confirmed by Harris (6). Further proof of the presence of glutamine in plasma has recently been provided by Archibald (2, 3) by the determination of ammonia liberated from plasma on treatment with dog kidney glutaminase.

Van Slyke and his associates (5) have recently demonstrated by experiments on dogs that the amide nitrogen of glutamine is the main source of urinary ammonia in acidosis.

That the large amount of ammonia which Ferdman, Frenkel, and Silakova (7) found liberated in urine by acid hydrolysis was derived from glutamine appears questionable because urea liberates significant amounts of ammonia by acid hydrolysis, and the amounts of urea in urine are so great that ammonia from this source might greatly exceed that from glutamine. This source of error has already been fully discussed (4, 6). There is no evidence that Ferdman, Frenkel, and Silakova eliminated this source of error from their analytical procedure as applied to glutamine determinations in urine or in blood. Enzymatic analysis has shown that insignificant amounts of glutamine are present in urine (2).

The values for plasma glutamine obtained by the quantitative ninhydrin- $\text{CO}_2$  method (4) are in good agreement with those reported initially by the writer (1) and later by Harris (6) and by Archibald (2, 3). The glutamine values for tissues confirm and extend those reported for dog tissues by Ferdman, Frenkel, and Silakova (7).

#### ANALYTICAL PROCEDURES

*Determination of Glutamine*—Glutamine was determined in deproteinized picric acid filtrates of plasma and tissues by the ninhydrin- $\text{CO}_2$  method described in the accompanying paper (4). In some plasma filtrates glutamine was also determined by measuring the ammonia nitrogen liberated on hydrolysis of filtrates with 2 N acid for 5 minutes at  $100^\circ$ ; hydrochloric acid was substituted for the sulfuric acid originally used by Krebs (20). The yield of glutamine amide nitrogen as ammonia is quantitative under these conditions. The nitrogen evolved as ammonia was measured by Archibald's micro technique for vacuum distillation and nesslerization (12). As recommended by Harris (6), ammonia liberated by hydrolysis of urea in a second 5 minute heating period was determined and subtracted from the ammonia nitrogen formed during the first 5 minute heating period. All values were determined in duplicate and were corrected for preformed ammonia in the filtrate.

For comparative purposes glutamine was also determined in some samples of plasma by the enzymatic glutaminase method of Archibald (2).

*Determination of Amino Nitrogen*—Amino nitrogen determinations were carried out in the Van Slyke-Neill manometric apparatus by the method of Van Slyke (11). Glutamine is anomalous in its reaction with nitrous acid as compared with asparagine and other amides in that it has previously been found to yield 80 per cent of its amide nitrogen in 4 minutes at  $22.5^\circ$  on treatment with nitrous acid; longer reaction time increases the yield by 1 or

2 per cent only (13). A sample of glutamine<sup>2</sup> (19.16 per cent N) on analysis by the Van Slyke nitrous acid procedure (11) gave 95 per cent of its total nitrogen as nitrogen gas in 5 minutes at 25°, equivalent to 84 per cent of the amide nitrogen, assuming the  $\alpha$ -nitrogen to react completely. All determinations reported in this paper were carried out at 25° with a 5 minute reaction time with nitrous acid. All amino nitrogen values of the solutions analyzed were corrected for their content of ammonia when it was present in significant amounts. Since 38 per cent of ammonia nitrogen is liberated as nitrogen gas with a 5 minute reaction time at 25° in the Van Slyke-Neill apparatus, 0.38 of the ammonia nitrogen, determined in a separate analysis, in the solutions analyzed was subtracted from the amino nitrogen values. All solutions that were to be subjected to analysis for amino nitrogen were rendered free of urea by treatment with urease as a preliminary step in the preparation of the filtrates, thereby eliminating the necessity for a correction due to its presence.

*Determination of Ammonia*—Ammonia nitrogen was determined in plasma filtrates by the micro vacuum distillation and nesslerization technique of Archibald (12).

#### EXPERIMENTAL

*Glutamine or Glutamine-Like Substances in Plasma*—The carboxyl nitrogen of a dialysate or a deproteinized picric acid human or dog plasma filtrate (to which is added phosphate buffer to give a solution of pH 6.5 and a final concentration of 0.08 M with respect to phosphate), heated for 90 minutes at 100° before analysis, is about 20 per cent lower than that for an unheated aliquot of the same filtrate. No further diminution of the carboxyl nitrogen occurs if the filtrate is heated longer than 90 minutes. That is, a 90 minute preliminary heating at 100° before analysis reduces the carboxyl nitrogen to a constant minimum value. Similarly the amino nitrogen of the filtrate determinable by the Van Slyke nitrous acid procedure (11) decreases to a constant minimum value; the ammonia nitrogen increases to a constant maximum value. All of these three forms of determinable nitrogen reach their constant values simultaneously. When acetic acid and sodium hydroxide are used instead of phosphate buffer to adjust the filtrate to pH 6.5, the carboxyl, amino, and ammonia nitrogen, determined on heated and unheated aliquot portions of the filtrate, are the same as those for filtrate samples containing phosphate, but the time necessary to heat at 100° to obtain constant minimum values is at least 4 hours.

The carboxyl, amino, and ammonia nitrogen of a filtrate without phosphate is illustrated in Fig. 1. Comparison of the curves for carboxyl

<sup>2</sup> The author is indeed grateful to Dr. G. W. Pucher and Dr. H. B. Vickery for a generous sample of pure glutamine.

nitrogen designated COOH-N (no phosphate) and COOH-N (phosphate 0.08 M) exemplify the effect of phosphate in reducing the time of heating at 100° necessary to obtain constant values (4).<sup>3</sup>

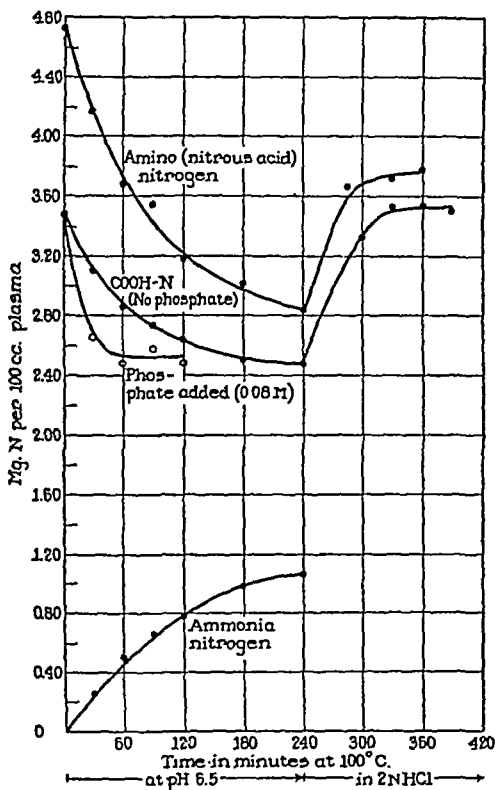


FIG. 1. Changes of carboxyl, amino, and ammonia nitrogen in picric acid filtrates of dog plasma on heating at 100° and on hydrolysis in 2 N hydrochloric acid.

In the experiment, results of which are embodied in Fig. 1, a single deproteinized picric acid filtrate of dog plasma, freed of urea and ammonia and adjusted to pH 6.5, was investigated. Phosphate buffer was omitted

<sup>3</sup> The significance attached to the effect of phosphate in decreasing the time necessary to heat at 100° at pH 6.5 to give maximum formation of pyrrolidonecarboxylic acid from glutamine is discussed in the accompanying paper (4). The fact that the rate of change of the carboxyl nitrogen of plasma filtrates is affected by the presence of phosphate (as compared with acetate of the same molar concentration and pH 6.5) in exactly the same way as solutions of pure glutamine are affected, is taken as additional evidence that the unstable amino acid in the filtrate behaves similarly to glutamine in this respect also.

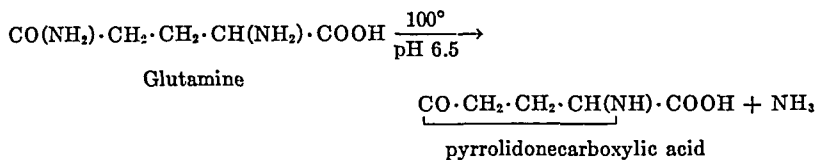


in order to take advantage of the slower change of the various nitrogen values on heating at  $100^{\circ}$  so that their parallel changes might be compared more exactly.

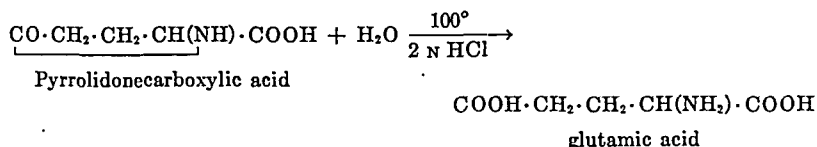
From Fig. 1 (the curve designated COOH-N (no phosphate)) the carboxyl nitrogen per 100 cc. of plasma for the unheated and heated aliquot samples of the filtrate is 3.48 and 2.48 mg. respectively. The difference between these values for this plasma represents the maximum loss of 1.00 mg. of carboxyl nitrogen per 100 cc. as a result of the preliminary heating at  $100^{\circ}$ . Similarly the amino nitrogen for the unheated and heated aliquot portions of the filtrate is 4.73 and 2.83 mg. respectively, a maximum difference of 1.90 mg. per 100 cc. of plasma. The ammonia nitrogen of the filtrate is zero and 1.04 mg. for the unheated and heated samples respectively, a net increase of 1.04 mg. per 100 cc. of plasma. The ratio of the increase in ammonia nitrogen to decrease in carboxyl nitrogen is  $1.04/1.00 = 1.04$ , the ratio of the decrease of amino nitrogen to the decrease in carboxyl nitrogen is  $1.90/1.00 = 1.90$ , and the ratio of the decrease of amino nitrogen to the increase in ammonia nitrogen is  $1.90/1.04 = 1.83$ . These ratios experimentally determined for a sample of glutamine of known purity were 1.02, 1.91, and 1.95 respectively.

A portion of the filtrate was heated at  $100^{\circ}$  for 4 hours at pH 6.5. This heated portion was subjected to hydrolysis at  $100^{\circ}$  in 2 N hydrochloric acid (final concentration) and the carboxyl nitrogen then determined. The carboxyl nitrogen of the hydrolysate was higher than that for an unhydrolyzed aliquot of the same heated filtrate. After an 80 minute period of hydrolysis the carboxyl nitrogen of the hydrolysate was the same, within the limits of experimental error, as that obtained initially. Prolongation of the hydrolysis caused no further significant increase. As illustrated (Fig. 1), the carboxyl nitrogen is 2.49 and 3.52 mg. for the heated unhydrolyzed filtrate, and the heated hydrolyzed filtrate, respectively, giving a net increase of 1.04 mg. per 100 cc. of plasma. Similarly the amino nitrogen is 2.83 and 3.75 mg., an increase of 0.92 mg. per 100 cc. of plasma. The ratio of the decrease in carboxyl nitrogen on heating, to the increase in carboxyl nitrogen on hydrolysis of the heated filtrate is  $1.00/1.04 = 0.96$ , and the ratio of the decrease in amino nitrogen on heating to the increase in amino nitrogen on hydrolysis of the heated filtrate is  $1.88/0.92 = 2.04$ . For glutamine these ratios experimentally determined were 0.96 and 2.00 respectively.

The changes in carboxyl, amino, and ammonia nitrogen of glutamine are a consequence of, first, the conversion of glutamine to pyrrolidonecarboxylic acid and ammonia on heating in solution at pH 6.5, and second, the hydrolysis of the pyrrolidonecarboxylic acid so formed on heating in 2 N hydrochloric acid to give glutamic acid. These reactions are quantitatively described by the following equations.



and



Quantitatively, within the limits of experimental error, the changes of carboxyl, amino, and ammonia nitrogen of plasma filtrates are identical with the changes observed in glutamine solutions. The effect of phosphate in accelerating the conversion of glutamine to pyrrolidonecarboxylic acid and ammonia has been demonstrated (4). The effect of phosphate on the unstable component in plasma filtrate is similar in all respects to the effect of phosphate upon glutamine. It is therefore reasonable to conclude from these data that the unstable component in human and dog plasma is, in fact, glutamine.

For the experiment reported in Fig. 1 fresh dog plasma was treated with urease of low amino acid content (24) to decompose urea, and a 1:6 deproteinized picric acid filtrate was prepared according to Hamilton and Van Slyke (25). The pH was adjusted to 9.8 to 10.0 and the ammonia removed by vacuum distillation at 20°. The solution was then adjusted to pH 6.5 by the addition of glacial acetic acid and made to its original volume with water. The filtrate, free of urea and ammonia, was divided into 50 cc. lots and placed in Erlenmeyer flasks, stoppered, and stored at -72° in a dry ice box till analyzed.

Aliquot portions of the filtrate were heated for 0, 30, 60, 90, 120, 150, 180, and 240 minutes at 100° in closed, partially evacuated all-glass reaction vessels (25). Carboxyl nitrogen, amino nitrogen, and ammonia nitrogen were determined on aliquot portions of these heated samples of filtrate.

For amino and carboxyl nitrogen values on heated, hydrolyzed filtrate the following procedure was adopted. A portion of filtrate was heated in a stoppered all-glass reaction vessel, partially evacuated, for 240 minutes until minimum amino and carboxyl nitrogen values were obtained. To aliquots of this heated filtrate concentrated hydrochloric acid was added to give a final concentration of 2 N; the aliquots were then heated in the 100° bath for 45, 90, and 120 minutes. After hydrolysis by this procedure the excess hydrochloric acid was removed by vacuum distillation to dryness. The dry residue was dissolved in water and made to its original volume.

Carboxyl nitrogen was determined on 5 cc. aliquot portions of the hydrolysate, reaction of amino acids with ninhydrin being carried out at pH 4.7 (4). The analytical values were corrected for conjugated  $\alpha$ -amino nitrogen of amino acids liberated from peptides and other conjugated forms by hydrolysis. The correction was made by subtracting 0.48 mg. of nitrogen per 100 cc. of plasma, found to be in the conjugated form (see below).

For amino nitrogen determinations, an aliquot of the hydrolysate was adjusted to pH 10.0 and ammonia was removed by vacuum distillation. The ammonia-free hydrolysate was then made up to its original volume with ammonia-free water. Analysis was carried out on 5 cc. samples. As in the case of carboxyl nitrogen, 0.48 mg. of nitrogen per 100 cc. of plasma was subtracted from the determined amino nitrogen values to correct for conjugated  $\alpha$ -amino nitrogen.

To determine the "conjugated"  $\alpha$ -amino nitrogen, a sample of unheated, unhydrolyzed filtrate was treated with ninhydrin at pH 4.7 for 10 minutes at 100° to decompose all free  $\alpha$ -amino acids. The excess ninhydrin was extracted from the solution with ethyl ether in a continuous extractor. The conjugated carboxyl nitrogen was not extracted by ether, nor was it detectable in the residual aqueous phase until after hydrolysis in 2 N hydrochloric acid (100° for 120 minutes). After hydrolysis the yield of carboxyl nitrogen on reaction with ninhydrin was found to be equivalent to 0.48 mg. of nitrogen per 100 cc. of plasma, the average of four separate determinations.

*Glutamine in Dog and Human Plasma*—Glutamine carboxyl nitrogen concentrations determined by the ninhydrin-CO<sub>2</sub> method (4) in dog and human plasmas, and, in a single sample of pig plasma, are given in Table I. Of the eighteen analyses of different dog and human plasmas, the glutamine carboxyl nitrogen in all but three examples constitutes 17 to 25 per cent of the total carboxyl nitrogen (Column 3). The amount of glutamine in the plasma varies from 4 to 20 mg. per 100 cc., and is higher in non-fasting than fasting human plasma.

For three samples of plasma the nitrogen liberated as ammonia from aliquot portions treated with dog kidney glutaminase was determined (3). The values obtained agree well with those obtained for glutamine carboxyl nitrogen by the ninhydrin-CO<sub>2</sub> method (4).

The ammonia nitrogen liberated by hydrolysis in 2 N hydrochloric acid for 5 minutes at 100° in a number of other samples was also determined. In general the ammonia nitrogen liberated under these conditions was found to be a little greater than the amount expected from the glutamine carboxyl nitrogen value of the same plasma; whether this may be due to other as yet unidentified labile sources of ammonia is being investigated.

*Glutamine and Amino Acid Carboxyl Nitrogen Values of Dog Tissues*—In Table II the glutamine carboxyl nitrogen of different tissues of the dog is compared with the total free carboxyl nitrogen of the same tissue; the amount of glutamine per 100 gm. of tissue (wet weight) is given in Column 4.

Heart muscle contains the largest amount of glutamine of any organ analyzed in the dog, with an average value of 225 mg. per 100 gm. of tissue, corresponding to approximately 56 per cent of the total free amino acids. The amount in diaphragm muscle is somewhat less, and in skeletal muscle still less. In general, however, glutamine was found to constitute

TABLE I

*Glutamine and Total Free  $\alpha$ -Amino Acid Carboxyl Nitrogen Values\* for Dog, Human, and Pig Plasma*

Subject	Amino acid carboxyl nitrogen			Gluta- mine	Amide nitrogen on hydrolysis		Remarks
	Total	Gluta- mine	$\frac{\text{Glutamine}}{\text{Total}} \times 100$ $\frac{(1)}{(2)} \times 100$		Enzy- matic†	Acid	
	(1)	(2)	(3)		(4)	(5)	
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
Dog 1	4.48	1.13	25.2	11.8			Fasting, before trauma
	8.04	1.92	23.9	20.0			“ after “
“ 2	4.49	1.12	25.0	11.7			“ normal
“ 3	3.74	1.17	31.3	12.2			“ “
“ 4	4.46	1.00	22.4	10.4		1.04‡	“ “
“ 5	4.19	1.46	34.8	15.2		1.48	“ “
“ 6	4.19	0.61	14.6	6.4	0.58		“ “
“ 7	4.54	1.02	22.5	10.6			“ “
“ 8	7.14	1.30	18.2	13.6			“ “
“ 9	5.26	1.32	25.1	13.8			“ “
Human 1	2.46	0.43	17.5	4.5	0.38		“ nephritic
“ 2	2.82	0.69	24.5	7.2			“ normal
“ 3	3.09	0.62	20.1	6.5			“ “
“ 4	3.74	0.74	19.8	7.7		0.84	“ “
“ 5	3.58	0.72	20.1	7.5		0.93	“ “
“ 6	5.19	1.18	22.7	12.3			Nonfasting, normal
“ 7	5.64	1.13	20.0	11.8			“ “
“ 8	4.28	0.88	20.6	9.2		0.97	“ “
“ 9	4.37	0.98	22.4	10.2		1.01	“ “
“ 1	5.14	0.60	11.7	6.3	0.59	0.69	Reconstituted from
“ 2	5.26	0.48	9.1	5.0			desiccated plasma§
“ 3	5.31	0.73	12.8	7.6		0.55	
Pig 1	5.31	0.63	11.9	6.6			Slaughterhouse blood

\* All analytical figures are the average of duplicate analyses.

† Enzymatic analysis kindly done by Dr. R. M. Archibald.

‡ Ammonia nitrogen liberated in 240 minutes at 100° and at pH 6.5.

§ Supplied through the courtesy of Dr. R. J. Westfall, Sharp and Dohme, Inc., Glenolden, Pennsylvania.

at least 50 per cent of the free amino acids in muscle tissue, a concentration at least twice that found in all other tissues analyzed.

TABLE II

*Glutamine and Free  $\alpha$ -Amino Acid Carboxyl Nitrogen Values\* of Normal Dog Tissues*

Organ tissue	Amino acid carboxyl nitrogen			Glutamine mg. per 100 gm.
	Total	Glutamine	$\frac{\text{Glutamine}}{\text{Total}} \times 100$	
	(1) mg. per 100 gm.	(2) mg. per 100 gm.	(3) per cent $\frac{(1)}{(2)} \times 100$	
Heart 1. Right ventricle.....	37.4	21.9	58.6	229
" 1. Left " .....	34.3	20.1	58.6	209
" 2.....	43.6	27.0	61.9	282
" 3.....	37.5	19.3	51.4	201
" 4.....	39.1	23.3	59.6	243
" 5.....	33.2	18.0	54.2	188
Average.....	37.5	21.6	55.7	225
Diaphragm 1.....	32.2	17.2	53.4	179
" 2.....	32.7	17.2	52.7	179
" 3.....	31.6	18.4	58.2	192
Average.....	32.2	17.6	54.6	183
Skeletal Muscle 1.....	25.1	9.2	36.6	96
" " 2.....	25.4	12.9	50.2	134
" " 3.....	25.1	12.2	48.6	127
" " 4.....	23.6	12.4	52.6	129
Average.....	24.8	11.7	47.0	122
Brain 1.....	39.5	8.4	21.3	87
" 2.....	24.4	6.5	26.6	68
" 3.....	22.4	5.2	23.2	54
" 4.....	15.3	5.6	36.6	58
" 5.....	13.9	5.0	36.0	52
Average.....	23.1	6.1	28.7	64
Liver 1.....	30.0	4.5	15.0	47
" 2.....	16.1	4.1	25.4	43
Average.....	23.0	4.3	20.2	45
Kidney 1.....	38.3	1.1	2.9	11.5
" 2.....	19.2	0.6	3.1	6.2
" 3.....	23.5	1.7	7.2	18.0
" 4.....	15.2	0.9	5.9	9.4
Average.....	24.0	1.1	4.8	11.3

\* All analytical values are the average of duplicate determinations.

TABLE II—Continued

Organ tissue	Amino acid carboxyl nitrogen			Glutamine (4) mg. per 100 gm.
	Total	Glutamine	$\frac{\text{Glutamine}}{\text{Total}} \times 100$	
	(1) mg. per 100 gm.	(2) mg. per 100 gm.	(3) per cent	
Stomach, fundus.....	19.5	3.5	17.9	36
"    pylorus.....	20.5	2.6	12.7	27
Small intestine.....	44.4	5.3	12.0	54
Large bowel.....	24.4	4.8	19.7	50
Spleen.....	31.3	5.9	18.8	61
Lung.....	14.6	1.9	13.0	20
Uterus.....	16.0	2.3	14.4	24

The small amount of glutamine found in kidney is noteworthy and is to be expected because of the presence of the enzyme glutaminase and the function which has been demonstrated for this enzyme system in the intact animal (5).

In confirmation of Van Slyke and Meyer (26), who used the nitrous acid method of Van Slyke (11) for the determination of free amino nitrogen in tissue filtrates, the free carboxyl nitrogen of tissue filtrates was found to be 5 to 10 times that of blood plasma.

#### DISCUSSION

In spite of the relative abundance of glutamine in plasma and tissues, there is little known concerning its physiological function. The early work of Thierfelder and Sherwin (17) in showing that phenylacetic acid is conjugated with glutamine and excreted in this conjugated form in the urine serves as a demonstration that glutamine is to be found in the body, rather than as evidence regarding its function in the normal animal. The demonstration by Van Slyke and his associates (5) that the amide nitrogen of glutamine is the main source of urinary ammonia is, however, direct evidence of a physiological function. Harris (6) has shown that the glutamine level of plasma in diabetic patients was significantly lowered by the administration of insulin. Harris did not determine that the administration of insulin altered the ratio of glutamine to total amino acids. In experiments of the writer on dogs (unpublished data) glutamine plasma levels have been observed to alter considerably but the ratio of glutamine carboxyl nitrogen to total carboxyl nitrogen remained unchanged, suggesting a shift of all amino acids from blood to tissues rather than a significant variation of glutamine concentration alone.

It is a privilege to acknowledge the author's indebtedness to Dr. D. D. Van Slyke for constant help throughout the course of this work. Thanks

are also due to Dr. R. M. Archibald for the glutaminase determination by his glutaminase method reported herein, and to Dr. R. A. Phillips for valuable help in procuring numerous blood samples and tissues.

#### SUMMARY

1. Experimental evidence is presented, based on the quantitative study of the relationships of carboxyl, amino, and ammonia nitrogen concentrations in plasma filtrates, before and after heating at 100° in solutions of pH 6.5, to show that there is an unstable amino acid in plasma filtrates, and that the unstable amino acid is identical with glutamine in all the reactions studied.

2. The normal concentration of glutamine in dog and human plasma varies from 6 to 12 mg. per 100 cc. and constitutes 18 to 25 per cent of the total free amino acid carboxyl nitrogen of the filtrates.

3. The concentration of glutamine has been determined in brain, liver, kidney, gastrointestinal tract, spleen, lung, uterus, and muscles of the dog.

4. The concentrations of glutamine were found to be greater in cardiac, diaphragmatic, and skeletal muscles of the dog than in the other tissues examined. Cardiac muscle contained the most, 225 mg. per 100 gm. Glutamine was found to provide 50 to 60 per cent of the total free amino acid carboxyl nitrogen of heart muscle.

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## FATTY ACID METABOLISM

### III. REACTIONS OF CARBOXYL-LABELED ACETIC ACID IN LIVER AND KIDNEY\*

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Recent studies with isotopic octanoic (1) and butyric acids (2) revealed that their transformation to ketone bodies in liver occurs by condensation of a 2-carbon intermediary (presumably acetic acid or a derivative thereof) formed by successive  $\beta$  oxidation of the fatty acid. The importance of acetate in the intermediary metabolism of fats and proteins is further emphasized by the demonstration, by Bloch and Rittenberg (3, 4), that isotopic acetyl groups are formed in the breakdown of a variety of fatty acids and amino acids labeled with deuterium. Though acetate has been found to take part in such anabolic reactions as the formation of acetoacetate (5), cholesterol (6), and fatty acids (7) in animals, and succinic acid (8), butyl alcohol (9), and citric acid (10) in lower organisms, little is known concerning its oxidative catabolism.

Inasmuch as the immediate oxidation products of acetate, *e.g.* glycolic and glyoxylic acids, are metabolized slowly if at all in tissues in which acetate is rapidly broken down (11), these substances are unlikely to be intermediates of acetate oxidation in animal tissues. It is more probable, therefore, that condensation reactions precede its ultimate degradation to  $\text{CO}_2$ .

In continuation of our investigation of the metabolic pathways of fatty acids, we are making a study of the oxidation of acetate in animal tissues, utilizing the stable carbon isotope,  $\text{C}^{13}$ , incorporated in the carboxyl group. The object of the present study was to elucidate the mechanism of the condensation of acetate to ketone bodies in liver, and to obtain information concerning the possible intermediate stages of acetate oxidation in kidney, which proceeds without the apparent formation of ketone bodies.

#### EXPERIMENTAL

*Preparation of Labeled Acetic Acid*—Acetic acid, labeled in the carboxyl group, was prepared by the action of methyl sulfate on 0.98 gm. (0.02 mole) of isotopic sodium cyanide according to the method of Walden (12) and subsequent hydrolysis of the acetonitrile by aqueous alkali. The

\* With the technical assistance of Mary Cammaroti, Lafayette Noda, and Ruth Wilson.

product was isolated as the sodium salt and its purity checked by determination of the Duclaux constants. The sodium cyanide contained 4.40 atoms per cent  $C^{13}$  and the sodium acetate contained 2.16 per cent, equivalent to a  $C^{13}$  content of 4.32 per cent in the carboxyl carbon atom.<sup>1</sup> The yield was 16.75 mm or 83 per cent based on the cyanide.

*Incubation with Liver Slices and Recovery of Products*—Approximately 1 mm of the labeled sodium acetate was added to 100 ml. of the buffer solution previously described (1) and a 30 ml. aliquot removed for acetate assay and determination of the Duclaux constants. 30 ml. aliquots were added to each of two 125 ml. Warburg type flasks, each containing 1.25 gm. of liver slices in the main compartment, alkali-soaked filter paper in the central well, and 2 N sulfuric acid in the side cup. All transfers were carried out in a nitrogen atmosphere to avoid contamination with atmospheric  $CO_2$ . The flasks were shaken in oxygen for 2 hours at 37.5°; the oxygen consumption was measured by a mercury-filled gas burette, attached to the two flasks by a T-connection. The acid was then introduced from the side cup and shaking continued 15 minutes to release the respiratory  $CO_2$ . The alkali-soaked papers were removed and treated as previously described for estimation and collection of the respiratory  $CO_2$ . The medium was separated from the tissue, combined, and divided in two portions. One portion was steam-distilled according to the procedure of Friedemann (13) and the distillate made alkaline and redistilled. The second distillate, thus freed of non-volatile substances and volatile acids, contains the acetone derived by decarboxylation of acetoacetate during distillation. It was isolated as the Denigès complex according to the procedure of Van Slyke (14). The residue of the second distillation, containing the sodium salts of the volatile acids, was acidified, distilled, and the Duclaux constants determined. It was finally redistilled and the acid exactly neutralized, phenolphthalein being used as an outside indicator. The neutral solution of the sodium salts was then evaporated to dryness.

The second portion of the medium was treated by the Van Slyke (14) procedure previously described (1) for decarboxylation of acetoacetic acid and isolation of the acetone and  $CO_2$ , after which the hydroxybutyrate was oxidized with dichromate and the acetone isolated.

The tissue was likewise divided in two portions, one of which was steam-distilled for recovery of any volatile acid which might have been bound by the tissue. The amounts so adsorbed were insignificant, however. The second portion of tissue was extracted successively with alcohol and ether, thereby separating the tissue into lipid and lipid-free fractions.

In the experiments with kidney slices the procedure was the same, except that the isolation of ketone body fractions was omitted, since trial

<sup>1</sup> The  $C^{13}$  contents are expressed as atoms per cent  $C^{13}$  excess, calculated from the equation, atoms per cent  $C^{13} = ((\text{moles } C^{13} \times 100) / (\text{moles } C^{12} + \text{moles } C^{13})) - 1.04$ .

experiments with normal acetic acid showed no trace of these substances. The non-volatile residue after distillation of the unutilized acetate was found to contain excess  $C^{13}$ . To purify this fraction further it was extracted continuously for 48 hours with ether. By this process all of the excess  $C^{13}$  was extracted, but since the amount of extract was only sufficient for a  $C^{13}$  determination, further identification was impossible.

TABLE I

*Distribution of Excess  $C^{13}$  in Products of Incubation of  $C^{13}$ -Labeled Acetic Acid with Liver and Kidney Slices from Fasted Rats*

	Liver				Kidney			
	Experiment 1, 2.50 gm. tissue		Experiment 2, 2.50 gm. tissue		Experiment 1, 2.00 gm. tissue		Experiment 2, 1.83 gm. tissue	
		$C^{13}$ excess		$C^{13}$ excess		$C^{13}$ excess		$C^{13}$ excess
	mm	atoms per cent	mm	atoms per cent	mm	atoms per cent	mm	atoms per cent
Acetic acid, start .	0.634	2.16	0.638	2.16	0.628	2.16	0.632	2.16
"    "    recovered	0.420	1.96	0.432	2.06	0.499	1.91	0.490	2.07
"    "    utilized..	0.214		0.206		0.129		0.142	
O <sub>2</sub> consumed	0.404		0.559		0.388		0.537	
CO <sub>2</sub> evolved in respiration	0.314	0.92	0.374	0.95	0.299	1.06	0.521	0.99
Acetoacetic acid	0.0750		0.0938		0		0	
$\beta$ -Carbon, direct		1.47		1.71				
"    by distillation		1.62		1.71				
Carboxyl carbon.		1.89		2.25				
$\beta$ -Hydroxybutyric acid .	0.0361		0.0277		0		0	
$\beta$ -Carbon		0.72		1.41				
Non-volatile carbon					1.13	0.05	0.89	0.10
"    "    ether					mg.	mg.		
extract ..					8	0.18	4	0.28
Tissue lipids. .		0.03		0.01				0.01
"    non-lipids		0.02		0.02				0.03

For the  $C^{13}$  determinations the solid fractions were converted to CO<sub>2</sub> by the Van Slyke and Folch wet combustion procedure (15). The fractions in solution were oxidized by a micro modification of the persulfate method of Osburn and Werkman (16).

### Results

*Ketone Body Formation in Liver*—In Experiment 1, the acetone, obtained as the Denigès complex formed *in situ* by the breakdown of acetoacetate in the presence of mercuric sulfate, contained 0.49 per cent  $C^{13}$ . This represents a  $C^{13}$  content in the carbonyl carbon of  $3 \times 0.49 = 1.47$  per cent (Table I). As a check on the specificity of this procedure, and to

avoid the possibility of inclusion of other carbon-containing substances, the precipitation with mercuric sulfate was employed on another portion of acetone, freed, by distillation, from non-volatile substances and volatile acids. This sample had a slightly higher  $C^{13}$  content, 0.54 per cent, representing 1.62 per cent in the carbonyl carbon. In Experiment 2, the  $C^{13}$  contents of the two acetone fractions were equal, 0.57 per cent, representing an excess of 1.71 per cent in the carbonyl carbon.

In both experiments the carboxyl carbon atoms had a somewhat higher  $C^{13}$  content than the corresponding  $\beta$ -carbon atoms. To verify this observation we carried out a third experiment (not included in Table I) with a new sample of labeled acetate containing 8.32 per cent  $C^{13}$  in the carboxyl group. In this experiment part of the acetoacetate was broken down by the procedure described and another portion was decarboxylated with aniline citrate, according to the method of Edson (17). Since the latter method does not require heating and is highly specific for  $\beta$ -keto acids, it is probably more reliable than heat decarboxylation. By this procedure we also found a somewhat greater  $C^{13}$  content in the carboxyl carbon than in the  $\beta$ -carbon, the respective values being 2.58 and 2.22 per cent. The results of this third experiment are somewhat ambiguous, however, in that the  $CO_2$  from heat decarboxylation had a  $C^{13}$  content of only 1.70 per cent.

The acetone derived by oxidation of  $\beta$ -hydroxybutyrate also contained excess  $C^{13}$ , a result to be anticipated considering the close metabolic relationship between acetoacetate and hydroxybutyrate. However, the calculated  $C^{13}$  content of the  $\beta$ -carbon atom of hydroxybutyrate was lower in each experiment than the corresponding value for acetoacetate. The quantitative significance of these differences is difficult to assess, in view of the possibilities of contamination by acetone or other substances arising by oxidation of substances other than hydroxybutyric acid.<sup>2</sup>

The  $C^{13}$  content of the carboxyl carbon of hydroxybutyrate could not be determined, since in the oxidation of hydroxybutyrate to acetoacetate the carboxyl  $CO_2$  was diluted by relatively large quantities of  $CO_2$  derived from other easily oxidizable substances present in the medium.

*Complete Oxidation of Acetic Acid in Liver*—In Experiment 1 the 0.314 mm of  $CO_2$  evolved by respiration contained 0.92 per cent  $C^{13}$ . Since the isotopic acetic acid had a  $C^{13}$  content of 2.16 per cent,  $0.92 \times 100/2.16$  or 42.5 per cent of the respiratory  $CO_2$  was derived by oxidation of isotopic acetate. This is equivalent to  $0.314 \times 0.425/2 \approx 0.067$  mm of acetate or  $0.067 \times 100/0.214 = 31$  per cent of the amount utilized. In Experi-

<sup>2</sup> In a third experiment the  $C^{13}$  contents of the acetone derived from acetoacetate and hydroxybutyrate were about equal, the respective values being 0.74 and 0.77 per cent.

ment 2, similarly calculated, 44 per cent of the respiratory  $\text{CO}_2$  came from the substrate, representing the complete oxidation of 0.082 mm or 40 per cent of the utilized acetate.

Comparison of the Duclaux constants of the acid before and after incubation reveals no significant formation of other volatile acids in the incubation procedure (Table II). However, a slight decrease in the  $\text{C}^{13}$  content of the unutilized acetate was observed, which seems to indicate a slight dilution by normal acetate formed during incubation. The quantity of preformed acetate in liver is negligible.

*Acetate Oxidation in Kidney*—In Experiment 1, 0.129 mm of acetate was utilized and 0.299 mm of  $\text{CO}_2$  was obtained, with a  $\text{C}^{13}$  content of 1.06 per cent. Hence,  $1.06 \times 100/2.16$  or 49.1 per cent of the total  $\text{CO}_2$  came from the substrate, representing the complete oxidation of  $0.299 \times 0.491/2 = 0.077$  mm or  $0.077 \times 100/0.129 = 59$  per cent of the utilized acetate.

TABLE II  
*Duclaux Constants of Volatile Acid Fractions*

	Per cent of total volume distilled						
	12.5	25	37.5	50	62.5	75	87.5
	Per cent of total acidity in distillate						
Normal acetic acid.	11.8	23.9	36.8	50.5	65.4	81.2	95.6
Isotopic " " . . . . .	12.0	24.4	36.1	49.0	63.7	79.2	93.9
After incubation, liver	14.7	26.3	38.2	50.9	64.8	79.8	93.8
" " kidney.	12.4	23.9	36.8	50.3	65.4	80.8	94.5

In Experiment 2, similar calculation reveals that 83 per cent of the utilized acetate was completely oxidized.

After removal of the acetate by distillation the non-volatile residue, containing 1.13 and 0.89 mm of carbon in the respective experiments, had a small  $\text{C}^{13}$  excess, all the excess  $\text{C}^{13}$  of which was extractable with ether. The amount of this fraction was so small, however, that only sufficient was available for a  $\text{C}^{13}$  determination. Consequently further identification was impossible.

As in the liver experiments, the Duclaux constants reveal that the recovered acetic acid was unaccompanied by other volatile acids, but there was similarly observed a small dilution by normal acetic acid, due either to acetate formation from tissue constituents during incubation or to preformed acetate.

*Recovery of  $\text{C}^{13}$* —As shown in Table III, the respiratory  $\text{CO}_2$  and ketone bodies account for 63 and 84 per cent of the  $\text{C}^{13}$  utilized as acetate in the

respective liver experiments. The  $C^{13}$  content of the non-volatile fractions was too low for an accurate estimation, but could not have represented more than 5 per cent of the total. The remainder was probably incorporated into the tissue by synthetic reactions. Although the tissue fractions did not contain significant  $C^{13}$  excesses, this does not signify that none of the unaccounted for  $C^{13}$  was incorporated in the tissue, as the small quantities involved would be masked by the relatively large mass of normal tissue carbon.

In the respective kidney experiments the respiratory  $CO_2$  and the non-volatile fraction account for 70.5 and 96.7 per cent of the total utilized  $C^{13}$ .

TABLE III  
*Per Cent of  $C^{13}$  Utilized As Acetic Acid, Appearing in Various Fractions*

	Liver		Kidney	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Acetoacetic acid.....	26.3	35.5	0	0
Hydroxybutyric acid.....	5.6	8.7	0	0
Respiratory $CO_2$ .....	31.2	39.5	59.8	82.5
Non-volatile substances.....	*	*	10.7	14.2
Tissue.....	0	0	0	0
Total accounted for.....	63.1	83.7	70.5	96.7

\* Not determined.

#### DISCUSSION

The equal distribution of excess isotope between the carbonyl and carboxyl carbon atoms of acetoacetic acid formed from isotopic octanoic acid (1) indicated that the metabolism of fatty acids in liver proceeds through the intermediary formation of a 2-carbon substance. The similar results reported herein with carboxyl-labeled acetic acid may be taken as additional confirmation of this postulation and further emphasizes the close relationship between acetic acid and this 2-carbon intermediary.

Of the total ketone bodies formed, only a part was derived from the isotopic acetic acid. This is indicated by comparison of the  $C^{13}$  contents of the respective substances. In Experiment 1, for example, the acetoacetate contained  $(1.62 + 1.89)/4 = 0.88$  per cent  $C^{13}$ , whereas the acetic acid had a  $C^{13}$  content of 2.16 per cent. If we assume that this difference represents dilution of the isotopic acetoacetate by non-isotopic acetoacetate derived from tissue constituents, we may calculate that  $0.88 \times 100/2.16 = 41$  per cent of the total acetoacetate came from the

substrate and 59 per cent from the tissue. Similar calculations for Experiment 2 indicate that 45 and 55 per cent were derived from the substrate and tissue, respectively. Though the origin of this non-isotopic acetoacetate is uncertain, the most probable source is the fatty acids of the liver lipids. If these fatty acids were broken down to acetic acid in the course of their conversion to ketone bodies, a dilution of the isotopic acetic acid would be expected.

Although a dilution was observed, it was not as large as expected. In Experiment 1, for example, 60 per cent of the 0.111 mm of ketone bodies came from the tissue. It should have required the production of  $0.111 \times 0.60 \times 2$  or at least 0.133 mm of acetic acid. Since 0.634 mm of acetate was originally present, the minimum expected dilution would be  $0.133 \times 100 / (0.634 + 0.133) = 17$  per cent, whereas the observed dilution was 9 per cent. In Experiment 2, the minimum dilution should again have been about 17 per cent, whereas the observed dilution was 11 per cent.

It should be pointed out that the expected dilution would be observed only if equilibrium exists between the biologically formed acetic acid and the isotopic substrate. If the rate of condensation is rapid in comparison with the diffusion, it is conceivable that the "biological" acetic acid, being produced intracellularly, is preferentially condensed before mixing with the main portion of extracellular isotopic acetate. This would be true, particularly, if the biological 2-carbon intermediary is not acetic acid itself, but a functional derivative such as acetyl phosphate, which would not be expected to diffuse readily.

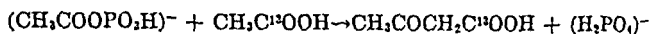
Two other observations point to the possibility that the biological intermediary is not acetic acid itself. First, the condensation of acetate does not occur in the absence of oxygen.<sup>3</sup> Under anaerobic conditions no acetate is utilized and no ketone bodies are formed. Since oxidation is not involved in the condensation of acetate to acetoacetate, it is conceivable that oxidative energy is required to convert acetate to an active form which can undergo the condensation. Lipmann (18) has brought forward evidence to show that the "active" acetate concerned in the many fold reactions of pyruvic acid is acetyl phosphate. This substance may be involved here also.

The second observation is the apparently greater concentration of  $C^{14}$  in the carboxyl than in the carbonyl group. This is difficult to explain on the basis of a condensation of identical molecules. If we assume, however, that the substrate molecules react, not only with one another, but also with a more active biological 2-carbon compound, the higher activity of the latter might be manifested by a greater tendency to act as the acetylating

<sup>3</sup> Unpublished observation.



agent in the condensation. With acetyl phosphate as an example, the reaction could be formulated as follows:



*Other Oxidation Pathways of Acetic Acid in Liver*—The  $\text{C}^{13}$  content of the respiratory  $\text{CO}_2$  represents the complete oxidation of 30 to 40 per cent of the utilized isotopic acetate. Inasmuch as acetoacetate is appreciably oxidized by fasted rat liver (19), part at least of the isotopic  $\text{CO}_2$  of respiration represents the breakdown of acetoacetate.

The question remains whether all of the isotopic  $\text{CO}_2$  represents breakdown of ketone bodies or whether acetate is metabolized by some other pathway. If all of the acetate goes through ketone bodies, the  $\text{C}^{13}$  content of the respiratory  $\text{CO}_2$  would be equal to or lower than the  $\text{C}^{13}$  content of the ketone bodies. If the  $\text{C}^{13}$  content of the respiratory  $\text{CO}_2$  were significantly higher than the ketone bodies, this would constitute proof for another oxidation pathway. In Experiment 1 the acetoacetate contained 0.88 per cent  $\text{C}^{13}$ , whereas the respiratory  $\text{CO}_2$  had 0.92 per cent; in Experiment 2, the respective values were 0.99 and 0.95 per cent; in a third experiment they were 1.20 and 1.21 per cent. Thus, in all three experiments the  $\text{C}^{13}$  contents were approximately equal. Inasmuch as we are reluctant to place too great reliance on small quantitative differences in view of the possibility of unknown sources of error in the  $\text{C}^{13}$  determinations, the above data are regarded as inconclusive as to whether or not acetate is oxidized through other pathways than acetoacetate. However, the approximate equality of the  $\text{C}^{13}$  contents of the acetoacetate and the  $\text{CO}_2$  suggests that perhaps all of the substances involved, whether from the substrate or tissue, pass through the ketone body stage prior to complete oxidation.

*Acetate Oxidation in Kidney*—The results of these experiments gave no conclusive information concerning the possible intermediates of acetate oxidation in kidney. The presence of  $\text{C}^{13}$  in the non-volatile material extractable with ether does suggest that possibly  $\text{C}_4$ -dicarboxylic acids or other components of the Krebs cycle may be involved. These possibilities are now being studied.

#### SUMMARY

1. The distribution of  $\text{C}^{13}$  in acetoacetic acid formed by condensation of acetic acid, labeled with  $\text{C}^{13}$  in the carboxyl group, indicates that the reaction proceeds by coupling of two acetyl groups.

2. Of the total acetoacetate, 41 to 45 per cent came from the isotopic acetate and the remainder presumably from constituents of the liver slices.

3. Although the results establish the ketone bodies as an intermediate

stage of acetate metabolism in liver, the data are inconclusive as to whether or not acetate is also metabolized by some other pathway not involving ketone body formation.

4. A decrease in the  $C^{13}$  content of the acetate recovered after incubation demonstrates the formation of acetate from tissue constituents.

5. Acetic acid is oxidized in kidney without the appreciable accumulation of intermediates. The presence of a small quantity of isotope in the non-volatile fraction extractable with ether suggests that the  $C_4$ -dicarboxylic acids or other components of the Krebs cycle may be intermediates of acetate oxidation in kidney.

We are greatly indebted to Dr. H. C. Urey and the Houdry Process Corporation of Marcus Hook, Pennsylvania, for their cooperation in this work.

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## A DIETARY FACTOR ESSENTIAL FOR GUINEA PIGS\*

### VI. CHANGES IN THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHORUS IN THE MUSCLE DURING A DEFICIENCY OF THE ANTISTIFFNESS FACTOR

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In diseases in which muscular activity is impaired because of a dystrophic condition, deviations from the normal concentrations of creatine phosphate and adenosine triphosphate can be expected. Reinhold and Kingsley (1) reported a decrease in the concentration of phosphocreatine and adenosine triphosphate in dystrophic muscles of guinea pigs deficient in vitamin E. The low level of creatine in the muscle of animals deficient in vitamin E was recognized by Goettsch and Brown (2) and has since been repeatedly confirmed (3-5). A correlation exists between muscular dystrophy and urinary excretion of creatine (6). These two phenomena are suggestive of a breakdown of creatine phosphate during the deficiency of vitamin E. Houchin (7) and Weissberger and Harris (8) found an increase in phosphate turnover during vitamin E deficiency. Changes in the concentrations of phosphocreatine and adenosine triphosphate in the brain have been reported in experimental poliomyelitis (9).

Guinea pigs raised on a diet mainly composed of skim milk to which the necessary minerals and known essential vitamins had been added developed a characteristic syndrome (10). The first outward sign of the deficiency was the development of a stiffness at the wrist joint. In the advanced stages of the deficiency the muscles were found to be extremely atrophied. Calcium deposits were found in many body tissues. Vitamin E, supplied either in the form of wheat germ oil or as the synthetic  $\alpha$ -tocopherol, did not cure or prevent the deficiency disease. The syndrome could, however, be prevented and cured by a factor present in raw cream (11) and several plant sources.<sup>1</sup>

It was found previously (12) that no creatinuria developed when guinea pigs were raised on a diet deficient in the antistiffness factor, but sup-

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† From a thesis submitted by one of the authors (H. L.) as partial fulfillment of the requirements for the degree of Master of Arts, Oregon State College.

<sup>1</sup> van Wagtendonk, W. J., and Wulzen, R., to be published.

plemented with  $\alpha$ -tocopherol. However, a more severe creatine excretion resulted when the diet was deficient in both  $\alpha$ -tocopherol and the anti-stiffness factor than when only  $\alpha$ -tocopherol was omitted. This indicates that the antistiffness factor has some influence in the creatine turnover. The concentration of the easily hydrolyzable phosphorus in the liver and kidneys of the experimental animals was lowered considerably during the deficiency (13). A high concentration of inorganic phosphate was found in these tissues. The level of inorganic phosphate in the blood of these animals was also significantly above normal (14). It might be concluded from these observations that a deviation from the normal levels of creatine phosphate and adenosine triphosphate in the muscle would result from a deficiency of the antistiffness factor. A significant decrease of the concentration of these compounds was found.

## EXPERIMENTAL

### *Method*

Guinea pigs were raised on the diet described by van Wagtendonk *et al.* (14). This diet consisted of skim milk powder and water to which the necessary minerals and known vitamins were added. The animals were bedded on autoclaved straw. The animals on the stock diet received rolled barley, greens, and straw *ad libitum*. At various intervals the guinea pigs were sacrificed. The animals were anesthetized with nembutal, and the musculus rectus femoris rapidly removed. The tissue was frozen at once in a mixture of dry ice and ethyl ether in order to prevent changes in the phosphate distribution (15, 16). After being weighed in the frozen state, the tissue was dispersed in 10 times its weight of ice-cold 10 per cent trichloroacetic acid by means of a Waring blender. The mixture was filtered immediately after maceration. The filtrate was made alkaline to phenolphthalein and the resulting solution analyzed for inorganic phosphate, creatine phosphate, and adenosine triphosphate according to the method of Stone (17). In order to determine the changes in the concentrations of inorganic phosphate, creatine phosphate, and adenosine triphosphate due to aging, three different age groups (13, 20, and 72 weeks) of animals on the stock diet were also analyzed. Wherever possible groups of fifteen animals were used.

### *Results*

The results are represented in Table I. The creatine phosphate and adenosine triphosphate concentrations increased slightly with progressive age, thus substantiating the findings of Fainshmidt *et al.* (18). The concentration of creatine phosphate in the musculus rectus femoris coincides with that found by Palladin and Epelbaum for the musculus biceps femoris (19).

Marked changes in the distribution of the acid-soluble phosphate in the muscle were found during the time limit of the deficiency period. The concentration of the inorganic phosphate increased as compared with that found in animals receiving a stock diet. Some fluctuation in the creatine phosphate and adenosine triphosphate content occurred in the early period of the deficiency. In the more advanced stages a significant lower level became established.

TABLE I

*Distribution of Acid-Soluble P in Muscle of Guinea Pigs Receiving Stock Diet and Skim Milk Diet*

Age	Stock diet				Deficient diet				
	No. of determinations	Mean, mg. per 100 gm.			Time on diet	No. of determinations	Mean, mg. per 100 gm.		
		Inorganic P	Creatine phosphate	ATP-ADP*			Inorganic P	Creatine phosphate	ATP-ADP*
<i>wks.</i>					<i>wks.</i>				
13	10	54.1	7.0	12.4	1	15	48.7	7.8	18.7
14					2	15	69.1	7.1	12.4
15					3	15	51.6	10.0	14.8
16					7	8	71.0	7.0	14.5
20					10	15	54.0	7.9	7.6
23	15	37.2	9.2	20.6	19	5	76.1	4.5	4.4
29					28	15	65.2	4.2	3.8
41					59	16	80.1	3.0	2.8
72									
	10	42.7	12.0	19.8					

\* ATP and ADP denote respectively adenosine triphosphate and adenosine diphosphate.

#### DISCUSSION

From the experiments reported here it becomes evident that the impaired function of the muscle during the deficiency of the antistiffness factors is correlated with a lowered content of compounds containing energy-rich phosphate bonds (20). This decrease of creatine phosphate and adenosine triphosphate in the muscle tissue parallels the drop in the easily hydrolyzable phosphate concentration in other tissues, *e.g.* liver and kidneys (13).

In a previous paper of this series (14) a high concentration of calcium was found in the muscle. The simultaneous presence of a high concentration of inorganic phosphate can account for the deposits of calcium phosphate found in so many tissues.

Although both a deficiency of vitamin E and a deficiency of the antistiffness factor result in a lowered concentration of creatine phosphate

and adenosine triphosphate in the muscle, a distinct difference is found in the fate of creatine. A deficiency of vitamin E is characterized by a severe urinary excretion of creatine. No creatine excretion was found when the diet deficient in the antistiffness factor was supplemented with  $\alpha$ -tocopherol. Animals raised on a diet deficient both in vitamin E and the antistiffness factor developed a creatinuria, more severe than when only  $\alpha$ -tocopherol was omitted (12). Patrick and Morgan (21) have reported that an unidentified fat-soluble factor, present in yeast and soy bean phosphatides, was necessary for the proper utilization of  $\alpha$ -tocopherol by the chick. No evidence for the need of this factor was found in our experiments.

### SUMMARY

A deranged distribution of the acid-soluble phosphorus in the muscle is found during a deficiency of the antistiffness factor. The concentration of inorganic phosphate increases during the deficiency, while the concentrations of creatine phosphate and adenosine triphosphate decrease after some fluctuations at the onset of the deficiency. The antistiffness factor and vitamin E have different functions in the animal organism.

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# THE INACTIVATION OF TYRAMINE BY HEART MUSCLE IN VITRO

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It has previously been shown (1) that rat heart slices and intestinal muscle strips can both deaminate the side chain of tyramine and cause the disappearance of hydroxy groups on the ring. Liver and kidney slices, on the other hand, cause only deamination. It was, therefore, of interest to determine whether one or more enzymes are responsible for the inactivation of tyramine by heart and to study the properties of the reaction. The function of the liver enzyme, presumably, is the inactivation of the amines formed in the intestine from amino acids by bacteria. In the heart, it may reasonably be assumed that the mechanism which causes the destruction of tyramine is also concerned with the inactivation of epinephrine or sympathin produced at the sympathetic endings. Support is given to this possibility by the fact that tyramine is inactivated by intestinal muscle in the same way as it is by heart, and by the fact that arterial muscle, as shown below, contains a similar enzyme system.

## EXPERIMENTAL

Heart muscle slices were made from the ventricle within 5 minutes of the removal of the heart from the animal. Approximately 120 mg. (wet weight) were suspended in 4.0 cc. of Krebs' bicarbonate-Ringer's solution, and shaken in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide at 37°. Suspensions of heart muscle were also made with a small Waring mixer. The particles were fine enough to pass through muslin. Under the same experimental conditions the slices and suspensions behaved in most respects in the same way. At the end of the experiment, 1.0 cc. of 20 per cent trichloroacetic acid was added, the precipitate centrifuged, and aliquots of the supernatant fluid were used for the estimation of the remaining amine groups by the Van Slyke method and the hydroxy groups by the method of Theis and Benedict (2). Standards for the latter determination were obtained by adding different amounts of tyramine to slices immediately before the addition of the trichloroacetic acid. Since in the Van Slyke apparatus, under our conditions, 40 per cent of the ammonia present comes off as nitrogen, it was necessary to correct for this by the use of the following equations:  $x + 0.4y = a$ , and  $x + y = b$ .



Then  $x = (a - 0.4b)/(0.6)$ , when  $x$  is the amine nitrogen present,  $y$ , the amount of ammonia,  $a$ , the nitrogen as measured by the Van Slyke method, and  $b$ , the amine nitrogen added at the beginning of the experiment. The equation is valid, for it was shown that ammonium salts added to heart slices under the experimental conditions can be quantitatively recovered at the end of the incubation period. Estimations of ammonia by distillation and subsequent nesslerization proved that the tyramine was deaminated. Table I shows that the deamination of tyramine occurs more rapidly than the removal of the hydroxy groups, and this is also true as the concentration of tyramine is altered.

In order to determine whether one or more enzymes are responsible for the destruction of tyramine by heart, the effect of a number of drugs and of oxygen tension was studied. It is well known (for literature *cf.* (3))

TABLE I

*Disappearance of Amine and Hydroxy Groups of Tyramine As a Function of Time and Concentration*

The time curve was obtained with rat heart suspension; the concentration curve with rat heart slices after 4 hours incubation.

Time	Time curve				Concentration curve		
	NH <sub>2</sub> -N disappeared from 0.164 mg. added as tyramine HCl		OH groups disappeared from 0.2 mg. added as tyramine HCl		Tyramine added	NH <sub>2</sub> -N disappeared	OH groups disappeared
min.	mg.	per cent	mg.	per cent	mg.	per cent	per cent
30	0.026	17	0.025	13	1.0	100	67
60	0.054	33	0.045	25	2.0	85	63
90	0.086	52	0.07	35	4.0	71	44
120	0.124	75	0.10	50			

that ephedrine, cocaine, methylene blue, and caprylic alcohol inhibit the amine oxidase of liver, and that this enzyme does not attack the hydroxy groups on tyramine. The effects of these drugs on the heart enzyme are shown in Table II, which also includes experiments with indole, indole acetic acid, and large concentrations of cyanide. All these drugs were tested on liver slices and on heart, and were found to cause approximately the same percentage inhibition. Indole and indole acetic acid interfere with the *p*-nitroaniline reagent so that values could not be obtained for their effect on the disappearance of the hydroxy groups. Ephedrine also interferes with the reagent, causing a cloudiness after 30 to 45 seconds. It is possible, however, to estimate the color during this period, with a standard which contains the same concentration of ephedrine. The methylene blue is almost quantitatively adsorbed onto the protein, and therefore does not interfere with the color. It is obvious that all the drugs,

with the exception of methylene blue, inhibit the deamination to a much greater extent than they do the disappearance of the hydroxy groups. This, however, does not necessarily mean that two enzymes are involved. The effect of oxygen tension is shown in Table III. Kohn (4) first showed that the activity of the amine oxidase of liver increases with increased oxygen tension. This is also true of the enzyme in heart, and both amine and hydroxy groups are affected in the same way. Weight for weight,

TABLE II

*Effect of Various Drugs on Disappearance of Amine and Hydroxy Groups of 2.0 Mg. of Tyramine Hydrochloride Incubated for 2 Hours with Rat Heart Slices*

Drug	NH <sub>2</sub> -N disappeared from 0.164 mg. added as tyramine HCl		OH groups disappeared from 0.2 mg. added as tyramine HCl	
	mg.	per cent	mg.	per cent
	0.139	85	0.140	70
$7.3 \times 10^{-3}$ M cocaine HCl	0.072	44	0.134	67
$5.9 \times 10^{-3}$ " ephedrine SO <sub>4</sub>	0.061	37	0.140	70
$2.0 \times 10^{-2}$ " caprylic alcohol	0.082	50	0.115	57
$1.3 \times 10^{-3}$ " methylene blue	0.044	27	0.014	7
$0.85 \times 10^{-2}$ " indole	0.057	35		
$0.57 \times 10^{-2}$ " indole acetic acid	0.082	50		
$0.5 \times 10^{-2}$ " sodium cyanide	0.070	43	0.130	65

TABLE III

*Effect of Oxygen Tension on Disappearance of Amine and Hydroxy Groups of 2.0 Mg. of Tyramine Hydrochloride after Incubation for 2 Hours with Rat Heart Suspension in Krebs' Phosphate Solution*

	NH <sub>2</sub> -N disappeared from 0.164 mg. added as tyramine HCl		OH groups disappeared from 0.2 mg. added as tyramine HCl	
	mg.	per cent	mg.	per cent
Air.	0.076	46	0.070	35
100 % oxygen	0.105	64	0.100	50

heart muscle deaminates tyramine at approximately the same rate as liver.

Thiourea, in a concentration of 2.5 mg. per cc., does not inhibit the disappearance of either the amine or hydroxy groups, which indicates that a copper-containing enzyme is not concerned in the reaction. It was observed, however, that the incubation of tyramine with heart slices causes a black pigment to accumulate on the slices. The formation of this pigment, which is not methemoglobin, is inhibited by thiourea and cyanide, and, curiously, increased by the presence of ephedrine. The amount of pigment formed must be very small, because the subsequent estimations

of the amine and hydroxy groups are not apparently affected by it. It seems probable that a small amount of the tyramine may be converted to melanin by the cytochrome oxidase system, just as epinephrine is converted to adrenochrome. Pigment formation was not seen, however, when liver slices were incubated with tyramine.

If tyramine is added to rat heart slices, no effect on the oxygen uptake is detectable (5). If it is added to heart suspensions in Krebs' phosphate solution, a rapid uptake of oxygen occurs. This is shown in Fig. 1. Unlike the amine oxidase of liver, however, the uptake does not stop when 1 atom of oxygen is taken up per molecule. This extra oxygen uptake is

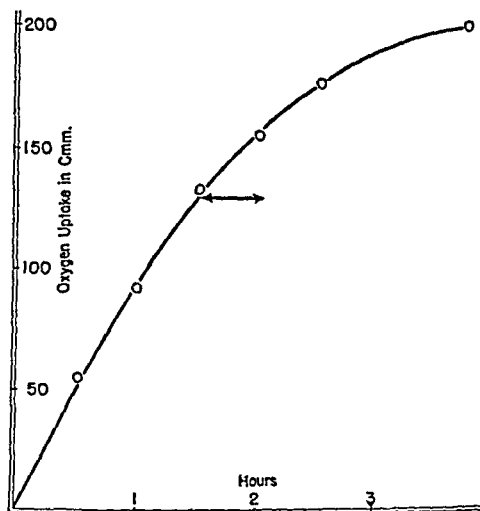


FIG. 1. The oxidation of 2.0 mg. of tyramine hydrochloride by rat heart suspension. The double arrow represents the theoretical oxygen uptake for 1 atom of O<sub>2</sub> per molecule.

probably caused by the oxidation of the hydroxy groups. That this is the probable mechanism for the attack on this group is indicated by the fact that autoclaving at 20 pounds pressure in strong acid after incubation fails to increase the amount of estimatable hydroxy groups. Therefore, conjugation does not occur. Cocaine, ephedrine, and indole inhibit the oxygen uptake of tyramine 26, 37, and 30 per cent respectively at 1 hour.

$\beta$ -Phenylethylamine is deaminated very slowly by rat heart slices. In three experiments, 2, 9, and 18 per cent of the amount added was deaminated in 2 hours, when at the same time 70 to 90 per cent of the tyramine amine groups disappeared. The same dry weight of liver slices deami-

nated 40 to 50 per cent of added  $\beta$ -phenylethylamine. Thus the enzyme in heart appears to be somewhat more specific. Mescaline added to rat liver slices is deaminated as indicated by the disappearance of the amine groups, and this is true to a smaller extent when it is incubated with heart muscle slices. It has previously been shown (6) that rat liver suspensions do not oxidize mescaline and heart suspensions are also inactive, which indicates that the intact cell structure is necessary for the oxidation of this amine in the rat, although this is not true for the rabbit. Possibly, as Blaschko (7) suggests, mescaline is oxidized by a separate enzyme.

Guinea pig and rabbit heart inactivate tyramine at about the same rate as rat heart, and the effect of drugs is similar. In the guinea pig heart the enzyme is present in equal concentrations at the apex and the base of the ventricles. On the other hand, dog and cat hearts are much less active. Even with 200 mg. of tissue present only 20 per cent or less of the added tyramine is inactivated in 2 hours. Both these animals have an active

TABLE IV

*Disappearance of the Amine and Hydroxy Groups of 2.0 Mg. of Tyramine Hydrochloride Incubated 3 Hours with 230 Mg. of Dog Thoracic Aorta and 130 Mg. of Rabbit Thoracic Aorta*

	NH <sub>2</sub> -N disappeared from 0.164 mg. added as tyramine HCl		OH groups disappeared from 0.2 mg. added as tyramine HCl	
	mg.	per cent	mg.	per cent
Dog.....	0.116	71	0.10	50
Rabbit.....	0.124	76	0.11	55

amine oxidase in the liver. The relative inactivity of their hearts is therefore puzzling, especially since the dog aorta is able readily to destroy both the amine and hydroxy groups of tyramine. This is shown in Table IV, which also includes the results with the rabbit aorta.

## DISCUSSION

The experiments with tyramine described above were designed to investigate the properties of an enzyme which may possibly be involved in the inactivation of epinephrine or sympathin produced at postganglionic sympathetic endings. Epinephrine is too unstable a molecule to work with *in vitro*. The ventricular muscle seemed the best tissue for this investigation, since Woolard (8), Nonidez (9), and others have shown that it is almost exclusively innervated by the sympathetic system, and physiologically this innervation is entirely made up of accelerator fibers. The presence of what may be called a modified amine oxidase in the ventricle, and also in the intestinal muscle and the aorta, is presumptive evidence that it may be concerned with epinephrine inactivation. But several

questions are raised by this assumption. First, why are the dog and cat hearts relatively inactive compared to those of the rat, rabbit, and guinea pig? Secondly, is the enzyme present in sufficient concentration to account for the relatively rapid disappearance of sympathetic effects? This is difficult to answer, for it is necessary to work *in vitro* with large concentrations of amine, which must diffuse to the site of the enzyme. 1.0 mg. of dry weight of rat heart under the experimental conditions inactivates, on the average, 6  $\gamma$  of tyramine per minute. This is much slower than the hydrolysis of acetylcholine by the cholinesterase, but in general sympathetic effects are more prolonged than effects mediated by acetylcholine. Thirdly, although cocaine and ephedrine inhibit the enzyme, why is it necessary to use such high concentrations? There is no answer to this question but it should be pointed out that the two other mechanisms suggested for the inactivation of epinephrine, namely, conjugation with sulfate or adrenochrome formation, are not inhibited at all by these drugs. Finally, does sympathetic denervation of the heart, which greatly increases its sensitivity to epinephrine, decrease the enzyme activity? It is hoped to answer this question in the near future.

#### SUMMARY

1. Rat, rabbit, and guinea pig ventricle slices inactivate tyramine by oxidation of both the amine and hydroxy groups. Dog and cat ventricles are much less active.

2. Cocaine, ephedrine, caprylic alcohol, and high concentrations of cyanide inhibit the deamination but have little effect on the oxidation of the hydroxy group. Methylene blue inhibits both and thiourea has no effect on either. Indole and indole acetic acid inhibit deamination, but their effect on the oxidation of the hydroxy group could not be determined. The oxidation of both groups is affected by oxygen tension.

3. Equal weights of rat ventricle and liver slices deaminate tyramine at approximately the same rate, but the liver enzyme, as previously shown, leaves the hydroxy group intact. The drugs inhibit the ventricle and liver enzymes to the same extent. Addition of tyramine to ventricular muscle suspensions results in an oxygen uptake greater than 1 atom of  $O_2$  per molecule, which suggests the oxidation of the hydroxy group or possibly the further oxidation of the ring. No conjugation of either amine or hydroxy group occurs.

4. In the guinea pig, the enzyme is present in the same concentration at the base and apex of the ventricle.

5. The dog and rabbit thoracic aorta inactivate tyramine in the same way as the ventricles.

6. The significance of the enzyme for the inactivation of epinephrine or sympathin is discussed.

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# STUDIES ON TRIMETHYLAMINE OXIDE

## I. OCCURRENCE OF TRIMETHYLAMINE OXIDE IN MARINE ORGANISMS\*

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Since 1909 when Suwa (1) showed the presence of hydroxytrimethylammonium hydroxide, commonly called trimethylamine oxide, in the muscle tissue of the shark (*Acanthias vulgaris*), several investigators (2-21) have found the base to occur widely in marine and fresh water organisms. However, no systematic study of the occurrence of the compound has been found in the literature. With the development of a simplified method of analysis based on previously suggested methods, a survey of the distribution of trimethylamine oxide was made possible and was carried out on biological materials available in the Puget Sound region.

### EXPERIMENTAL

Lintzel (22) devised a method for the determination of trimethylamine oxide based upon the reduction of the compound by means of Devarda's alloy in dilute hydrochloric acid. He found that choline, betaine,  $\gamma$ -butyrobetaine, and carnitine did not interfere.

In confirmation of Lintzel's work, the reduction of trimethylamine oxide in normal hydrochloric acid in the presence of Devarda's alloy (0.5 gm. per 5 ml. of solution) was found to be complete in 10 minutes at 105°, 15 minutes at 95°, and 35 minutes at 80°. Choline, betaine, methylguanidine, creatine, tyrosine, cystine, glycine, and proteins were found not to interfere. The specificity of the method used in the present work was checked by isolating trimethylamine oxide from one source from which it had not been previously reported.

In the analysis of marine organisms, a weighed sample of tissue or organism was finely ground in a mortar and transferred with distilled water into a graduated 50 ml. centrifuge tube. The material was extracted for at least 2 hours at approximately 10° with frequent stirring. After centrifuging, the total volume was noted, and the supernatant liquid decanted. This solution was used for the determination of trimethylamine and trimethylamine oxide.

\* Taken from a thesis presented by George J. Benoit, Jr., as partial fulfillment of the requirements for the degree of doctor of philosophy, University of Washington, 1942.

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Trimethylamine was determined by steam distillation at 30° under reduced pressure in an all-glass distillation apparatus described elsewhere (23). An aliquot of the tissue extract was diluted to 5 ml. in the distilling flask, and the solution neutralized if necessary with N sodium hydroxide. It was distilled for 10 minutes into 2 ml. of 0.02 N sulfuric acid, after the addition of 0.8 ml. of formalin and 2 ml. of 20 per cent sodium carbonate. The excess sulfuric acid was titrated with carbonate-free 0.006 N sodium hydroxide, with chlor-phenol red as the indicator. The difference between this titration and that of a blank represented trimethylamine plus traces of other volatile bases carried over under the conditions of the experiment.

In the determination of trimethylamine oxide, an aliquot of tissue extract was diluted with distilled water to 5 ml., and 1 ml. of 6 N hydrochloric acid was added. The oxide was reduced by digestion for 15 minutes at 95° with 0.5 gm. of Devarda's alloy. After reduction the cooled solution was quantitatively transferred to the distilling flask. The solution and washings, amounting to about 8 ml., were neutralized with 6 N sodium hydroxide. The distillation was carried out as described above after addition of 0.8 ml. of formalin and 2 ml. of 20 per cent sodium carbonate. The titration gave total trimethylamine after reduction, from which the preformed trimethylamine was subtracted. The sensitivity of the method varies with the amount of sample available, but in general was found to be near 0.1 micromole of trimethylamine oxide per gm. of tissue.

The distribution of trimethylamine oxide is given in Table I. In many cases several values are given to show the range observed for a given species. The distribution in the blood and various tissues of elasmobranch and teleost fish is also given to show the marked difference between them. The values given for invertebrates are for muscle tissue if not otherwise specified; in the case of small animals the entire animal was used.

Diatoms and marine algae were analyzed and found to be free of trimethylamine and trimethylamine oxide. The algae analyzed were *Nereocystis lutekeana*, *Porphyra naiadum*, and *Ulva lactuca*, which represent the three phyla, Phyophyceae, Rhodophyceae, and Chlorophyceae respectively.

Five sea anemones of the tribe Hexactiniae of the phylum Coelenterata were analyzed and found to contain from 5.7 to 27.0 micromoles of trimethylamine oxide per gm. of body weight. Segmented worms of the families, Terebellidae and Nereidae of the phylum Annulata were found to contain no trimethylamine oxide.

#### *Isolation of Trimethylamine Oxide*

The only positive proof of the presence of a base such as trimethylamine oxide in any organism is the isolation of some recognizable derivative and

TABLE I

*Distribution of Trimethylamine Oxide in Some Marine Animals\* (in Micromoles Per Gm. of Moist Tissue)*

## Invertebrates

## Molluscoidea

*Terebratalia transversa* (branchiopods)..... Negligible

## Echinodermata

*Strongylocentrotus franciscanus* (urchin)..... "

*Cucumaria miniata* (sea-cucumber)..... "

*Stichopus californicus* (sea-cucumber)..... "

## Mollusca

## Pelecypoda

*Ostrea japonica* (Pacific oyster)..... "

*Mytilus edulis* (mussel)..... "

*Paphia staminea* (clam)..... "

*Saxidomus giganteus* (clam)..... "

*Macoma inquinata* (clam)..... "

*Cardium californiense* (cockle)..... 13, 11

" *corbis* (cockle)..... 23

*Pecten hercicus* (scallop). Muscle..... 46, 50, 39, 52, 48, 41, 45, 52,  
32

" " Organs..... 4.6, 4.0, 2.2, 2.3

" *hindsii* (scallop). Muscle..... 77, 30, 56, 38, 71, 51, 38

" " Organs..... 8, 5, 9

" *jordani*. Muscle..... 32

## Amphineura

*Katherina tunicata* (black chiton)..... Negligible

*Cryptochiton stelleri* (giant chiton)..... "

## Gastropoda

*Anisodoris nobilis* (sea slug)..... "

*Thais lamellosa* (snail)..... "

*Littorina sitchana* (snail) (entire animals)..... "

## Cephalopoda

*Polypus hongkongensis* (octopus)..... 17

*Loligo opalescens* (squid)..... 107, 111

## Arthropoda (Crustacea)

## Copepoda (entire animals)

Mixture; largely *Corycaeus affinis*, *Calanus finmarchius*, *Tortanus discaudatus*, and *Epidabiocera amphrites*..... 45

Mixture; nearly completely *Corycaeus affinis*..... 16

## Cirripedia

*Balanus cariosus* (barnacles) (entire animal)..... 17

" *nubila* (barnacles)..... 52, 63, 42, 71

Amphipoda sp. (sand-flea) (entire animals)..... 2.2

## Decapoda

*Pandalus danae* (shrimp)..... 48, 28, 63

*Pagurus alaskensis* (hermit crab)..... 26

" *ochotensis* " "..... 47

" *setosis* (hermit crab)..... 46

TABLE I—Concluded

## Invertebrates—continued

<i>Pagurus tenuimanus</i> (hermit crab).....	30, 28
<i>Oregonia gracilis</i> (spider crab).....	13
<i>Pugettia</i> " " " .....	9
<i>Cancer gracilis</i> (crab).....	22
" <i>productus</i> " .....	46
<i>Hemigrapsus nudus</i> (shore crab).....	11

## Vertebrates

## Elasmobranch fish

*Squalus suckleyi* (dogfish). Muscle 135, 88, blood serum 64, kidney 42, liver 8, 18, pancreas 73, spleen 62, stomach 86

*Hydrolagus collicii* (ratfish). Muscle 121, 134, blood serum 7.5, 6.5

## Teleost fish

*Sebastes* sp. (rock fish). Muscle 16, 40, 46, 38, 48, heart 21, skin 7, liver 0.7, 0.8, blood serum, eggs, kidney, gill-rakers, spleen, stomach, intestine all negligible

*Scorpaenichthys marmoratus* (bull cod). Muscle 46; blood serum negligible

*Platyonectidae* sp. (flounders). Muscle 20, 21, 19, blood serum negligible

*Roccus saxatilis* (sea bass). Muscle 44

*Taeniotoxa lateralis* (blue perch). Muscle 48

*Oncorhynchus kisutch* (silver salmon). Muscle (adults caught in salt water) 6.2, 6.5, (adult spawning fish) 8.3

*Oncorhynchus tshawytscha* (king salmon). Muscle (fresh water fingerling) 0-0.6, (adults caught in salt water) 7.1, 8.3, (adult spawning fish) 2.1

\* We wish to thank Professor Trevor Kincaid for having supplied us with the names of many of the organisms studied. We wish also to thank Mr. Richard T. Smith of the Washington State Department of Fisheries and Professor Lauren R. Donaldson for having procured some to the specimens used.

its identification. Because of the time required the isolation and characterization of trimethylamine oxide were confined to *Pecten* muscle.

An aqueous extract of 1.6 kilos of adductor muscle of *Pecten hercynicus* was deproteinized with tannic acid in the presence of phosphoric acid. The clear filtrate was neutralized with sodium hydroxide and evaporated under reduced pressure on the water bath. The residue was extracted repeatedly with methanol at 60-65°, the insoluble portion being discarded.

The methanol filtrate was evaporated to a syrup which was repeatedly extracted with 96 per cent ethanol. The base was precipitated from the extract as the picrate by addition of a saturated solution of picric acid in ethanol. After recrystallization from ethanol, the crystals were dried over calcium chloride.

The melting point of trimethylamine oxide picrate is reported in the literature to be in the range 196-202°, with decomposition. The authors found the melting point of pure trimethylamine oxide picrate prepared from

alcoholic solution to be 198–199°,<sup>1</sup> that of the picrate from *Pecten* 199–201°, and the mixed melting point 196–197°, all melting with decomposition.

Part of the trimethylamine oxide picrate from *Pecten* was converted to the aurichloride. The gold value indicated the salt to be analytically pure hydroxytrimethylammonium aurichloride.

$(\text{CH}_3)_3\text{NOH} \cdot \text{AuCl}_4$ . Calculated, Au 47.5; found, 47.4

#### DISCUSSION

Trimethylamine and trimethylamine oxide were not found in marine algae or in diatoms.

Of the invertebrates studied only one phylum, namely Arthropoda, of the class Crustacea, showed trimethylamine oxide in every species analyzed.

Of the Mollusca, trimethylamine oxide was not found in the species analyzed of Gastropoda or Amphineura. It was found in Cephalopoda, in which it had been previously reported. Pelecypoda showed occasional occurrence. It was present in all specimens of *Pecten* and *Cardium* analyzed. It was not found in the oysters, mussels, or clams analyzed. The base was isolated from the muscle of the *Pecten* and shown to be trimethylamine oxide.

Of the phylum Chordata, tunicates and fish were analyzed. Tunicates did not show an appreciable amount of trimethylamine oxide. Both elasmobranch and teleost marine fish contain the oxide in their muscles; however, only the elasmobranchs had measurable amounts in the blood.

The above findings raise the interesting and as yet unsolved problem as to the origin and metabolic function of trimethylamine oxide. The occurrence in all marine arthropods, even in copopods, would indicate that it is of endogenous origin and has some fundamental function in the metabolism of this group. Of the other invertebrates and vertebrates in which it is found occasionally the question arises as to whether it is endogenous, formed in the metabolism of the animal, or entirely of exogenous origin, being obtained from the food. While no hard and fast rule can be formulated, it appears that the herbivorous animals living on algae or phytoplankton or carnivorous animals living on herbivorous animals do not contain appreciable amounts of trimethylamine oxide, while those living upon zooplankton (copopods) or other crustaceans or upon crustacean eaters do contain trimethylamine oxide.

#### SUMMARY

Trimethylamine oxide was found to be of general occurrence in marine Crustacea. Occurrence in other marine organisms is reported.

<sup>1</sup> All melting points are corrected.

In contrast to the results obtained with elasmobranch fish, the blood of marine teleost fish contained no appreciable trimethylamine oxide.

Trimethylamine oxide was isolated from the muscle tissue of *Pecten hericius*.

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## STUDIES ON TRIMETHYLAMINE OXIDE

### II. THE ORIGIN OF TRIMETHYLAMINE OXIDE IN YOUNG SALMON\*

By GEORGE J. BENOIT, JR.,† AND EARL R. NORRIS

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(Received for publication, October 16, 1944)

Trimethylamine oxide has been found widely distributed in marine fish; however, its origin or its function in metabolism is not known. Hoppe-Seyler (1) found it in the muscle, blood, and urine of elasmobranchs along with high concentrations of urea and suggested that it played a rôle similar to that of urea. He considered it to be a weakly basic, non-toxic end-product of nitrogen metabolism which assisted in maintaining osmotic pressure and water balance. The fact that trimethylamine oxide is not found in the blood of marine teleost fish (2), in contrast to the relatively high concentration in the blood of elasmobranchs, may suggest a different function in metabolism.

Beatty (3) analyzed muscle extracts of eel (*Anguilla rostrata*) from individuals living in fresh water and also from those found in brackish water, and found appreciable quantities of trimethylamine oxide only in the latter.

The trimethylamine oxide content of the muscle tissue of fresh water fish has been found to be negative or very low. This is true also of the muscles of young salmon while they are still in fresh water. Adult salmon taken from salt water contain about 8 micromoles of trimethylamine oxide per gm. of tissue.

The greater amount of trimethylamine oxide in the muscle tissues of salmon in salt water might be of endogenous origin and associated with the change of osmotic requirements with environment, or might be derived from exogenous sources.

If the trimethylamine oxide is of endogenous origin, the transference of young salmon from fresh to salt water while they are maintained on a trimethylamine oxide-free diet should give rise to an increase of the base in the muscle tissue. In the following experiment such a test was carried out.

\* Taken from a thesis presented by George J. Benoit, Jr., as partial fulfilment of the requirements for the degree of doctor of philosophy, University of Washington, 1942.

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## EXPERIMENTAL

Twenty-five 1 year-old Chinook salmon (*Oncorhynchus tshawytscha*) were placed in two connected tanks, each of 40 liters capacity. The water was circulated by a small centrifugal pump which forced it through a sand filter, and was aerated constantly.

The temperature of the water was maintained at about 10° by means of cooling coils.

The water was changed from fresh water to the salinity of sea water in a stepwise manner, the volume fraction of salt water being increased by one-eighth about every 5 days. At each change of salinity the tanks were

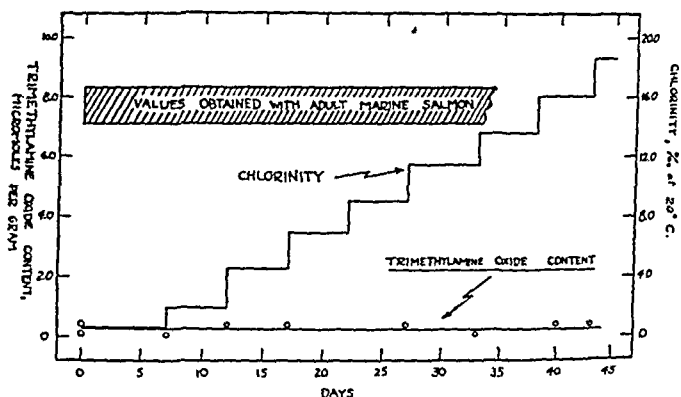


FIG. 1. Trimethylamine oxide concentration in young salmon and the chlorinity of the environmental water during the transition from fresh to sea water. The shaded bar represents values of trimethylamine oxide concentration in muscle tissue of adult Chinook salmon caught in salt water.

thoroughly cleaned. When the salinity was equal to that of sea water, the fish were transferred to an aquarium with circulating salt water maintained at approximately 10°. The salinity of the water was determined at each step by the Mohr method for total halide. During this period, the diet of the fish consisted of ground fresh beef liver.

Specimens taken periodically for analysis were killed and the heads, viscera, and tails removed. The carcasses were ground and analyzed in the manner described previously (2).

## Results

The variation of the trimethylamine oxide content of the fish carcasses plotted against time, and the change in the concurrent chlorinity of the environmental water plotted against time, are given in Fig. 1.

After the fish had been in sea water for 8 days, they were divided into two groups and placed in separate tanks. One group was continued on the liver diet, and the other was fed a diet which consisted of 50 per cent liver and 50 per cent of some trimethylamine oxide-containing material (salmon meal for 1 week, then ground *Pecten hericius* muscle tissue). At weekly intervals a fish was removed from each tank and analyzed for trimethylamine oxide content. Results are given in Fig. 2.

Throughout the experimental period the fish appeared to be in excellent condition.

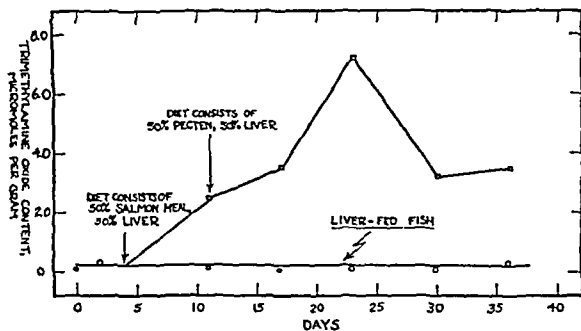


Fig. 2. Trimethylamine oxide concentration in young salmon on different diets

#### DISCUSSION

During the period of increasing salinity there was no significant variation in trimethylamine oxide content of the young salmon on the liver diet; even after they had been in sea water for 5 weeks they showed no increase. It is assumed that there would not have been an increase over a longer experimental period, but the results show that an increase in the trimethylamine oxide of the tissues over that present while the fish are in fresh water is not necessary for normal activity of young Chinook salmon either encountering or in salt water.

On the other hand, when the young salmon were fed trimethylamine oxide-containing material, there was a rapid accumulation of the compound in the muscle tissue. The concentration in the tissues attained that present in the normal marine adults after 3 weeks on the diet.

These results strongly suggest that, at least in the case of salmon, the occurrence of trimethylamine oxide in large amounts in the muscle tissue is due to the accumulation of ingested trimethylamine oxide, rather than to an endogenous metabolic function.

The reason for the decline in trimethylamine oxide content after the 3rd week on the trimethylamine oxide-supplemented diet (Fig. 2) probably lies



in the feeding habits of salmon. Isolated salmon will eat but little if at all. The last two fish on the trimethylamine oxide-containing diet did not feed well.

#### SUMMARY

When the environment of young Chinook salmon was changed from fresh to sea water while the fish were on a trimethylamine oxide-free diet, there was no significant change in the trimethylamine oxide concentration in their muscle tissues.

When young Chinook salmon in salt water were fed a diet which contained trimethylamine oxide, some of the compound was retained in their tissues.

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# STUDIES ON TRIMETHYLAMINE OXIDE

## III. TRIMETHYLAMINE OXIDE EXCRETION BY THE RAT\*

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(Received for publication, October 16, 1944)

The feeding of choline, betaine, trimethylamine, or trimethylamine oxide to mammalian animals has been reported to give rise to an increased urinary excretion of trimethylamine or trimethylamine oxide (1-9), although some workers failed to find such an increase (10, 11). Trimethylamine and trimethylamine oxide are excreted rapidly and nearly quantitatively, largely as the oxide.

Wünsche (12) found that intestinal bacteria could split trimethylamine from trimethylamine oxide and choline, but only in traces from betaine. Müller (8) ascribed the increased trimethylamine excretion he found from choline-fed dogs to this source.

If the trimethylamine or trimethylamine oxide which arises following the feeding of choline is due solely to absorption and excretion of the products of intestinal bacterial action on this compound, then differences in excretion should be encountered, depending on whether the compound is given by mouth or injected.

The urinary excretion of trimethylamine oxide following the feeding and injection of choline, trimethylamine oxide, and related compounds was studied in the albino rat.

### EXPERIMENTAL

Rats 28 days of age were put in individual metabolism cages with screen bottoms, so designed that the urine and feces might be collected separately. The basal diet and distilled water were administered *ad libitum*. A basal diet low in trimethylamine and trimethylamine oxide was prepared from casein 18, corn-starch 58, Hawk-Oser<sup>1</sup> salt mixture 4, dried yeast 10, cod liver oil 2, and Crisco 8 per cent. The diet was found to have no detectable trimethylamine or trimethylamine oxide by the method used.

The compounds studied were prepared in solutions of such a strength

\* Taken from a thesis presented by George J. Benoit, Jr., as partial fulfillment of the requirements for the degree of doctor of philosophy, University of Washington, 1942.

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<sup>1</sup> Hawk, P. B., and Oser, B. L., *Science*, 74, 369 (1931).

that 0.1 or 0.2 ml. contained the desired amount to be administered. When given orally, the sample was diluted with milk. Injections were made intraperitoneally.

The urine was preserved with sulfuric acid during collection. The 48 hour specimens following the administration of the compound to be studied were analyzed without delay for trimethylamine and trimethylamine oxide by the method previously described (13), 5 ml. portions being used for each analysis.

The relatively high concentration of ammonium salts as contrasted with those present in extracts of fish, etc., necessitated the use of 1.3 ml. of formalin per 5 ml. of urine and decreased the reproducibility of results to  $\pm 0.02$  ml. of 0.006 *N* sodium hydroxide. A blank obtained by distilling solutions of the same concentration of c.p. ammonium salts in distilled water was subtracted from the titrations for urine.

The results indicate that the order of magnitude of trimethylamine in the normal urine of both the rat and man is much lower than that of trimethylamine oxide. This confirms the conclusion of Erdmann (14), who could find no trimethylamine in fresh normal human urine. From his results and the similar ones of Takeda (15), it is probable that the trimethylamine from time to time reported to be present in human urine (16-18, 9) resulted either from bacterial or other reduction of part of the trimethylamine oxide present or from the decomposition during the analytical procedure of closely related compounds which occur normally in urine. Evidence for reduction was found by several workers (15, 5, 9).

The experiments were conducted over an 8 week period when the animals were 10 to 18 weeks of age.

Controls, which were fed the quantity of milk used for the oral ingestions, or into which were injected 0.2 ml. portions of distilled water, showed no change in trimethylamine or trimethylamine oxide excretion.

### *Results*

The results of these experiments are given in Table I, expressed in mean excretion plus and minus the average deviation from the mean. "Zero" means less than 0.04 micromole per ml.

In order to study further the excretion of trimethylamine and trimethylamine oxide following injection of trimethylamine hydrochloride, each of twelve rats was injected intraperitoneally with 49 micromoles of this substance. Analyses were made on pooled urine samples collected at intervals of 6 and 12 hours for 48 hours. The results are given in Table II.

At the conclusion of these experiments, the rats were divided into two groups. One group was fed 8 to 10 gm. of codfish each day for a week, the other the same amount of beefsteak. The animals were provided basal

diet and distilled water *ad libitum*. The rats were then killed by chloroforming and the muscles excised and analyzed. The urine from the rats of each group was pooled and analyzed at the end of the experiment.

TABLE I

*Urinary Excretion of Trimethylamine and Trimethylamine Oxide by Young Albino Rats Following Feeding or Injection of Choline, Betaine, Trimethylamine Hydrochloride, and Trimethylamine Oxide*

Experiment No.	No. of animals	Compound administered	Amount	Method of administration	Excretion per rat, per 48 hrs.		
					Trimethylamine	Trimethylamine oxide	Trimethylamine + trimethylamine oxide
			micromoles		micromoles	micromoles	micromoles
1	12	Basal diet only			0	3 ± 1	3 ± 1
2	10	Choline chloride	112	Feeding	0	33 ± 5	33 ± 5
3	12	" "	112	Injection	0	5.5 ± 2.5	5.5 ± 2.5
4	12	Betaine	114	Feeding	0	5 ± 2	5 ± 2
5	10	" "	114	Injection	0	4 ± 2	4 ± 2
6	11	Trimethylamine oxide	113	Feeding	4 ± 2	67 ± 7	71 ± 7
7	12	" "	113	Injection	4 ± 1	76 ± 11	80 ± 10
8	8	Trimethylamine hydrochloride	49	Feeding	5.5 ± 2	30 ± 6	35 ± 7
9	12	" "	49	Injection	11 ± 3	26 ± 8	38 ± 10

TABLE II

*Urinary Excretion of Trimethylamine and Trimethylamine Oxide by Young Albino Rats Following Intraperitoneal Injection of 49 Micromoles of Trimethylamine Hydrochloride into Each of Twelve Rats, As Function of Time Since Injection*

The results are for pooled urine.

Time after injection	Excretion per time interval		
	Trimethylamine	Trimethylamine oxide	Trimethylamine + trimethylamine oxide
hrs.	micromoles	micromoles	micromoles
0-6	69	195	264
6-12	14	61	75
12-24	3.7	54	58
24-36	0	33	33
36-48	0	12	12
Total.....	87	355	442

This experiment would test the reaction of the animals to a normal ingestion of trimethylamine oxide and other bases found in fish and of cho-

line in meat. Also, a determination of the trimethylamine oxide concentration in the muscle tissue of the animals on the fish diet would indicate whether or not there was any accumulation of the compound in the muscle tissue, as there is in marine fish (19).

The codfish used assayed at the beginning of the experiment 0.9 micromole of trimethylamine per gm. and 43 micromoles of trimethylamine oxide per gm.

The group on the fish diet ingested 17,000 micromoles of trimethylamine oxide and 360 micromoles of trimethylamine, calculated on the basis of no further reduction of trimethylamine oxide.

Table III gives the urinary excretion of trimethylamine and trimethylamine oxide of the two groups.

Kapeller-Adler and Krael (20) claimed to have shown the presence of trimethylamine oxide in mammalian muscle to the extent of 8 mg. per cent

TABLE III

*Urinary Excretion of Trimethylamine and Trimethylamine Oxide by Rats on Meat and on Fish Diet Supplements*

The values represent the 1 week collection; six rats in each group.

Diet	Excretion		
	Trimethylamine	Trimethylamine oxide	Trimethylamine + trimethylamine oxide
	micromoles	micromoles	micromoles
Meat.....	0	590	590
Fish.....	1900	12,600	14,500

(1.1 micromoles per gm.). We could not confirm their results by our method of analysis; a determination on beefsteak indicated a concentration of trimethylamine oxide less than 0.04 micromole per gm. Analysis of the muscle tissue of 4 week-old rats showed that if any trimethylamine oxide was present its concentration was less than 0.07 micromole per gm. The concentration of trimethylamine oxide in muscle tissue of the rats which had been on either the meat diet or the fish diet for 1 week was less than 0.04 micromole per gm., indicating that there was no accumulation of the compound in the muscle tissue after ingestion of rather large amounts of trimethylamine oxide.

#### *Isolation of Trimethylamine and Trimethylamine Oxide from Rat Urine*

As the method of determination of trimethylamine and trimethylamine oxide is based on the non-specific titration of a volatile base, the only proof of the presence of the bases is the actual isolation and identification of some known derivative.

The small amount of trimethylamine oxide present in the urine of the rats on the basal diet with no supplements was not conducive to its separation and identification. Instead, the trimethylamine resulting from its reduction was isolated.

4 liters of the reduced urine were treated with formalin (250 ml. per liter of urine) and sodium carbonate solution. It was then steam-distilled at 30° under reduced pressure into excess sulfuric acid. After being redistilled several times, the base was precipitated as the aurichloride and recrystallized from absolute alcohol.

$(\text{CH}_3)_3\text{HNAuCl}_4$ . Calculated, Au 49.5; found, 49.3

600 ml. of the filtered accumulated urine of the rats on the fish diet, as described above, were acidified with sulfuric acid and freed of some impurities by two partial precipitations with phosphotungstic acid. The resulting filtrate was treated with excess barium chloride and rendered slightly alkaline by the addition of sodium hydroxide. The filtrate was steam-distilled at 30° under reduced pressure for 5 minutes to remove ammonia and trimethylamine. The barium was then removed with carbon dioxide and the neutral filtrate concentrated *in vacuo* on the water bath to a thick syrup. The residue was extracted with methanol; after filtration, the filtrate was evaporated to a thick syrup *in vacuo* on the water bath. This process was then repeated three times with absolute ethanol. The filtrate from the last extraction was treated with saturated absolute alcoholic mercuric chloride to complete precipitation of the trimethylamine oxide. The precipitate, separated by centrifuging, was dissolved in water and decomposed with hydrogen sulfide. The clear filtrate was concentrated on the water bath to a small volume and the trimethylamine oxide was precipitated by the addition of 30 per cent chloroauric acid in 2.5 N hydrochloric acid. The crystalline precipitate was recrystallized from 5 N hydrochloric acid.

$(\text{CH}_3)_3\text{NOHAuCl}_4$ . Calculated, Au 47.5, found, 47.5

#### DISCUSSION

When choline was fed, a noticeable increase of urinary trimethylamine oxide was found. However, there was no evidence that injected choline was converted to trimethylamine oxide. There was practically no difference in trimethylamine oxide excretion following the feeding and injection of betaine. These results tend to confirm the conclusion that bacterial action in the intestine is responsible for the formation of trimethylamine or trimethylamine oxide, followed by excretion as trimethylamine oxide.

Both ingestion and injection of trimethylamine oxide and trimethylamine gave rise to the excretion of some trimethylamine. When trimethylamine

was injected, some was rapidly excreted *per se*, perhaps before it could be oxidized. Trimethylamine oxide was at least partly reduced, since some excretion as trimethylamine occurred. The site of the reduction other than bacterial reduction in the intestine may be the liver, as Ackermann, Poller, and Linneweh (21) have shown that mammalian liver *in vitro* is capable of reducing trimethylamine oxide in part to trimethylamine.

The small amounts of trimethylamine oxide excreted by animals fed the basal diet alone as well as the increased excretion when the diet was supplemented with beefsteak can probably be attributed to dietary choline.

#### SUMMARY

There was a noticeably greater excretion of trimethylamine oxide when choline was fed to albino rats than when the same amount was injected intraperitoneally. This suggests that the trimethylamine oxide arose from the action of intestinal bacteria on choline.

When trimethylamine and trimethylamine oxide were fed to or injected into rats, the compounds were rapidly and nearly quantitatively excreted, largely as the oxide.

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# THE HEMATOPOIETIC ACTIVITY OF XANTHOPTERIN IN YOUNG SALMON

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(Received for publication, November 14, 1944)

Since the first report from this laboratory of the hematopoietic activity of xanthopterin when administered to young salmon (1) several papers have appeared (2-7) suggesting a possible rôle of xanthopterin in nutrition and especially linking it with folic acid, vitamin M, and possibly vitamin B<sub>12</sub>.

There is considerable difference in the nutritional requirements of various types of animals. When young salmon and trout are fed synthetic diets which are adequate for the rat, the fish become anemic and the mortality is high. Ascorbic acid does not improve the diet, but the addition of 20 to 40 per cent of fresh liver renders the diet adequate for fish (8). Phillips (9) suggested that anemia be used as an index of factor H (10) deficiency; however, this factor is probably complex and made up of more than one component. The following experiments demonstrate the rôle of xanthopterin in alleviating fish anemia.

## EXPERIMENTAL

The young salmon used in these experiments were reared in hatchery ponds on a diet of 20 per cent liver and 80 per cent spawned-out salmon. The fish in each pond showed a considerable variation in erythrocyte count and contained a fairly large proportion of more or less anemic fish. The variation between members of the same pond was attributed largely to the individual's ability to obtain natural food in the form of insects. In spite of the great variation in erythrocyte count of fish taken at random from a pond, groups of fish having a uniform erythrocyte count could be selected from each pond on the basis of gill color. Table I gives representative results obtained by taking a group at random from such a pond and also the values obtained on groups selected according to gill color. The random group, C, Table I, gives every third value from a group of 60 fish with actual counts which ranged from 50,000 cells per c.mm. to 1,200,000 cells per c.mm., with a standard deviation of 300,000 cells per c.mm. Selection by gill color gave more uniform groups of animals with standard deviation of approximately 30,000 cells per c.mm.

To show the hematopoietic effect of xanthopterin on young salmon, two



lots of 9 month-old Chinook salmon (*Oncorhynchus tshawytscha*), hatched from eggs taken in the fall of 1940, were selected according to gill color. One of the lots had an average count of 800,000 cells per c.mm. (Lot A) and the other 1,300,000 cells per c.mm. (Lot B). The fish of Lot A were divided into groups and placed in four troughs, those of Lot B into two troughs. All the troughs were screened so that insects would not be available to the fish. The water temperature during the experiments

TABLE I  
*Individual Erythrocyte Counts (Cells per C.mm.) on Young Chinook Salmon*

Group C, taken at random	Group G. R., selected by gill color	Group I. L., selected by gill color	Group I. H., selected by gill color
1150 $\times 10^3$	690 $\times 10^3$	740 $\times 10^3$	1470 $\times 10^3$
920	740	820	1180
510	670	880	1300
120	780	840	1270
990	680	730	1310
100	720	760	
1200	690	770	
1100	760	850	
890	680	860	
470	730	820	
310		770	
500		830	
220		790	
310*		780	
350		740	
480		770	
190		810	
100		830	
420		790	
790		840	

ranged from 10–13°. The experimental fish were fed ground spawned-out salmon.

Three levels of xanthopterin (10, 20, and 40 mg. per kilo of body weight) were injected into three of the groups of Lot A, the fourth group being kept for a control. One of the groups of Lot B was injected with 20 mg. of xanthopterin per kilo of body weight and the second kept for a control. Erythrocyte counts were made on the 1st, 2nd, 3rd, 5th, 8th, and 14th days after injection. The results are given in Fig. 1. Because of the small size of the fish, the animals must be sacrificed in obtaining the blood for the cell count. Each point on the curves represents the mean of enough determinations to reduce the standard error of each mean to 30,000 cells.

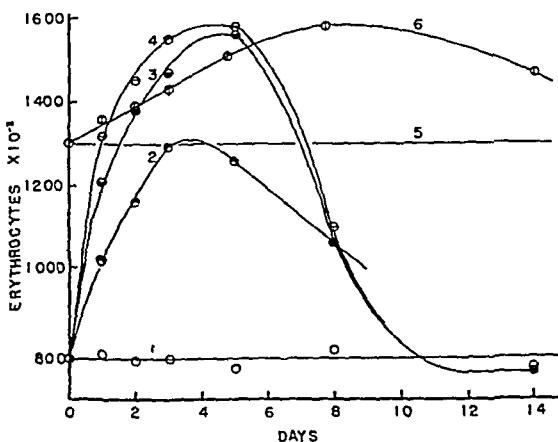


FIG. 1..Postinjection erythrocyte counts on young Chinook salmon injected with synthetic xanthopterin. Curve 1, controls for Lot A, no injection; Curve 2, group of Lot A, injected with 10 mg. of xanthopterin per kilo of body weight; Curve 3, group of Lot A, injected with 20 mg. of xanthopterin per kilo of body weight; Curve 4, group of Lot A, injected with 40 mg. of xanthopterin per kilo of body weight; Curve 5, controls for Lot B, no injection; Curve 6, group of Lot B, injected with 20 mg. of xanthopterin per kilo of body weight.

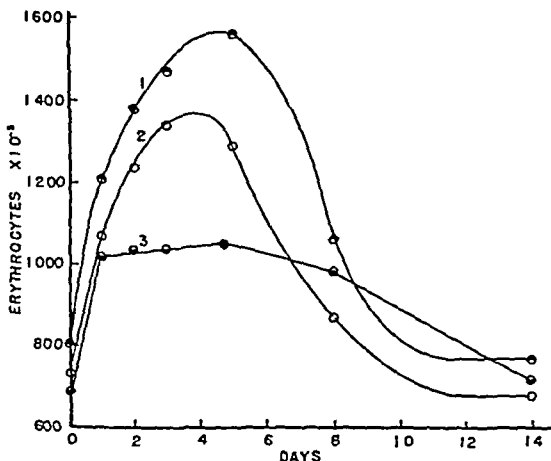


FIG. 2. Postinjection erythrocyte counts on young salmon of different species injected with 20 mg. of xanthopterin per kilo of body weight. Curve 1, Chinook salmon (*Oncorhynchus tshawytscha*); Curve 2, silver salmon (*O. kisutch*); Curve 3, sockeye salmon (*O. nerka*).

The response of groups of Lot A on different levels of xanthopterin showed a correlation between the amount injected and the rise in cell count. The highest recorded counts approach a maximum value between 1,550,000 and 1,600,000 cells per c.mm. Approximately the same maximum value was reached when 20 mg. per kilo were injected into the fish of Lot B.

A number of erythrocyte counts were made on apparently normal wild salmonoid fish, some caught in fresh and others in salt water. There was considerable variation among the wild fish; however, the highest values did not exceed the maximum values obtained in the above experiments. It appears that values between 1,500,000 and 1,600,000 cells per c.mm. might be considered as a normal maximum of optimum cell count. Cell counts were made on wild Chinook salmon, silver salmon, and steelhead trout.

In order to test the effect of xanthopterin on different species of salmon of approximately the same age, lots of silver salmon (*Oncorhynchus kisutch*) and sockeye salmon (*Oncorhynchus nerka*) were selected according to gill color and run parallel with the experiment on Chinook salmon. All the conditions of the experiment were kept the same. The two lots of fish were each divided into two troughs. One group of each lot was injected with 20 mg. of xanthopterin per kilo of body weight and the other kept for a control. Fig. 2 shows the relative response obtained with the three species of salmon, starting with approximately the same cell count and receiving 20 mg. of xanthopterin per kilo of body weight. All three species gave a rapid rise in cell count following injection. However, the response varied with the species; Chinook salmon gave the greatest and sockeye the least increase. The sockeye salmon were slowest in returning to the original value.

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#### SUMMARY

1. Young experimental fish of relatively uniform erythrocyte count may be selected from a random sample of a pond by gill color;
2. Xanthopterin has a hematopoietic action on young Chinook salmon, which is correlated with the dosage of xanthopterin used.
3. A maximum cell count of approximately 1,600,000 was not exceeded regardless of the dosage of xanthopterin or the initial cell count of the group of fish.
4. A species variation in response to injected xanthopterin was observed

between three species of the genus *Oncorhynchus*: Chinook salmon, silver salmon, and sockeye salmon.

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# THE USE OF DIAZOTIZED *p*-AMINOACETOPHENONE IN THE DETERMINATION OF VITAMIN B<sub>6</sub> (PYRIDOXINE)

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Swaminathan (1), in 1940, published a chemical method for the determination of pyridoxine, with diazotized sulfanilic acid as the color reagent for pyridoxine. The results obtained by this method were in good agreement with data published by a bioassay method of Waisman and Elvehjem (2). The procedure used to free the extracts of interfering substances was, however, long and difficult to perform. Bina, Thomas, and Brown (3) published an improved chemical procedure for the determination of vitamin B<sub>6</sub>, with diazotized sulfanilic acid as the color reagent. These authors employed alkaline ethyl alcohol as a selective solvent which aided in the purification and served to stabilize the color reaction.

A new diazo reaction for pyridoxine is described in this paper. The reagent is diazotized *p*-aminoacetophenone. The alkaline modification of this reagent was first used by Prebluda and McCollum (4) for the determination of thiamine. In the method reported here the diazo reagent is used for the reaction with pyridoxine immediately after the sodium nitrite treatment, without subsequent treatment with alkali. This reagent produces a good color with pyridoxine and is more sensitive and specific than diazotized sulfanilic acid as previously described by Swaminathan and the present authors. This sensitivity, we believe, is due to the fact that the color reaction is produced at a pH of 7.0 to 7.3 instead of at the higher pH of 10 to 11 which is necessary when diazotized sulfanilic acid is employed as the color-producing reagent. Solutions containing from 0.5 to 2.0  $\gamma$  of pyridoxine per ml. are readily measured by this procedure. While the specificity and sensitivity of this reagent are increased over that of diazotized sulfanilic acid, the stability of the color is not as good. That is, the color develops to a maximum within 2 to 5 minutes, after which it gradually fades. The time interval, however, between maximum color development of the complex and the start of fading is ample to permit of accurate reading.

The use of diazotized *p*-aminoacetophenone instead of sulfanilic acid in the color development permits the employment of synthetic resins instead of sodium tungstate in the purification process. The substitution of this simple adsorption step with Amberlite for the complicated technique and

voluminous precipitates involved in the sodium tungstate treatment materially simplifies the method.

### *Preparation of Reagents*

*Solution I*—3.18 gm. of *p*-aminoacetophenone are dissolved in 45 ml. of concentrated hydrochloric acid, and then diluted to 500 ml. with distilled water. This solution is kept in a glass-stoppered volumetric flask and stored in the ice box when not in use. Under these conditions the solution is stable for at least 2 months. Prebluda and McCollum (4) found it stable for at least 6 months.

*Solution II*—4.5 gm. of sodium nitrite are dissolved and made up to a final volume of 100 ml. with distilled water.

*Solution III*—2.0 ml. of Solution I are measured into a 25 ml. brown, glass-stoppered graduate, placed in an ice bath, and then 2.0 ml. of Solution II are added. The solution is kept in the ice bath for 10 minutes with occasional whirling, after which 8.0 ml. more of Solution II are added. The graduate is allowed to stand an additional 20 minutes with occasional whirling of the tube. With the completion of this reaction the diazo reagent is ready for use. This reagent should be used within an hour after preparation.

*Solution IV*—Alkaline ethyl alcohol; made by diluting 2 ml. of a 25 per cent NaOH solution to 100 ml. with 95 per cent ethyl alcohol.

*Solution V*—Acetic acid solution; 8.0 ml. of glacial acetic acid diluted to 100 ml. with distilled water.

*Solution VI*—Acetate buffer, pH 4.5; 54.4 ml. of glacial acetic acid and 111 gm. of hydrated sodium acetate crystals dissolved and diluted to 1000 ml. with distilled water.

*Solution VII*—Sodium acetate solution; 50 gm. of hydrated crystals dissolved and diluted to 100 ml. with distilled water.

*Solution VIII*—A standard solution of pyridoxine containing 100  $\gamma$  per ml. This solution is made slightly acid (pH 4.5) with sulfuric acid and kept in a brown glass flask in the ice box. A daily working standard containing 10  $\gamma$  per ml. is prepared from this solution.

### *Color Development*

The color development step proceeds as follows: To 10 ml. of an alcoholic solution at pH 7.0 to 7.3 containing from 5 to 25  $\gamma$  of pyridoxine are added 2.0 ml. of distilled water, followed by 2.0 ml. of 50 per cent hydrated sodium acetate (Solution VII), and then 1 ml. of diazo reagent (Solution III). The contents of the reaction graduate are mixed by inverting the cylinder after the addition of each reagent. After the addition and mixing of the sodium acetate solution, the cylinder is let stand for a few seconds to allow all the air bubbles to escape from the solution.

Then 1 ml. of the diazo reagent is allowed to run slowly down the side of the reaction cylinder, the formation of air bubbles being avoided. The contents are again mixed gently. The 1 ml. pipette used for the addition of the diazo reagent is carefully rinsed with distilled water after each use. This technique of the color reaction is important and should be followed carefully.

The time required for the color to develop to maximum intensity is approximately 3 to 5 minutes after the addition of the diazo reagent and it is stable for at least 2 minutes. This point is determined by color intensity measurements during the development. The instrument used to measure the yellow color is a Pfaltz-Bauer fluorophotometer, in which is employed a combination blue and yellow filter with maximum transmission at about 420  $m\mu$ . The instrument is adjusted to 0 extinction or 100 per cent transmission with a blank consisting of all the reagents except pyridoxine. The color developed in the blank is quite stable for a period of 1 hour, but the color developed with pyridoxine begins to fade slowly after reaching a maximum intensity within 5 to 6 minutes after adding the diazo reagent.

#### EXPERIMENTAL

A sample containing from 100 to 200  $\gamma$  of pyridoxine is weighed into a 125 ml. Erlenmeyer flask and 50 ml. of sulfuric acid (0.1 N) are added. The mixture is autoclaved at 15 pounds excess pressure for 30 minutes, cooled, and then incubated at 40° for 2 hours in a buffered solution containing 0.4 gm. of equal parts of taka-diastase and papain. The buffered solution is prepared by dissolving 0.2 gm. each of taka-diastase and papain in 10 ml. of acetate buffer solution of pH 4.5 (Solution VI). The hydrolysate is diluted to 100 ml. with distilled water and filtered through No. 1 Whatman paper. 0.5 gm. of Amberlite resin, No. IR-4, analytical grade, is added to the clear filtrate to adsorb soluble interfering substances to prevent their adsorption on the Super Filtrol. A 40 ml. portion of the resin-free extract is pipetted into a glass-stoppered centrifuge tube containing 0.6 gm. of Super Filtrol. This mixture is allowed to stand for 30 minutes with occasional whirling, and then centrifuged. The liquid is decanted and discarded. The Super Filtrol is washed with 40 ml. of distilled water and again centrifuged and the washing discarded. 20 ml. of alkaline ethyl alcohol (Solution IV) are added to the tube containing the Super Filtrol. The tube is placed in a water bath held at 60–65° for 30 minutes with occasional whirling, then cooled, and centrifuged. The alcoholic solution of the pyridoxine is then decanted into a 50 ml. beaker and the residue washed with 10 ml. of alkaline alcohol, centrifuged, and the alcohol added to the 50 ml. beaker.

The combined extract is adjusted to pH 7.3 with acetic acid solution



(Solution V) and diluted to 40 ml. with alcohol. 10 ml. portions are used for the pyridoxine determinations with the diazo reagent. The machine is set to zero extinction, or 100 per cent transmission by means of a complete reagent blank. This blank is made by carrying the reagents through the entire process, 10 ml. of the final solution being used. After diazo treatment the machine is set by this blank for use on the sample. The color developments are as previously described. The solutions used and referred to by number are prepared as described previously.

Determinations of pyridoxine were made on a number of biological materials. The diazo reagent was employed for a comparison with diazotized sulfanilic acid (Table I). The method employed for extracting the naturally occurring pyridoxine and freeing the extracts of interfering

TABLE I  
*Pyridoxine Content of Some Food Materials*

Material	Pyridoxine		
	p-Amino-aceto-phenone	Sulfanilic acid	Values from literature
	$\gamma$ per gm	$\gamma$ per gm	$\gamma$ per gm
Dried brewers' yeast, Sample I	64.7	64.5	39-40 (1), 55 (2)
" " " " II	75.2	75.6	
" " " " III	67.8	68.0	
" beef liver	86.4	80.0	100 (4)
Rice bran concentrate	135.0	137.0	
Whole wheat flour	7.2	10.1	
Barley malt	12.8	11.9	4.7-4.8 (1), 4.6 (5)
Yeast extract (liquid)	160.0	148.0	

substances is a modification of our procedure previously published in this *Journal* (3).

#### DISCUSSION

The strength of acid used in preparing the sample for enzymatic digestion is greater than previously used by us for this purpose. This increased strength of acid used does not give higher values of pyridoxine in the materials tested but prepares the material to better advantage for the purification step with Amberlite. Experiments were carried out in which different strengths of acid were used for the hydrolysis up to 2 N sulfuric acid without improvement. The use of higher strengths of acid proved detrimental because of increased breakdown of protein products that affect the purification process. The hydrolysis with 0.1 N sulfuric acid, without subsequent enzymatic digestion, gave results comparable

with those followed by enzymatic digestion. However, enzymatic digestion is especially desirable with products containing starch and is advised for all products.

The removal of interfering compounds is more readily and easily accomplished by the use of Amberlite IR-4 than by the more complicated sodium tungstate precipitation procedure. This material does not adsorb pyridoxine under the conditions used in this procedure but does remove material that would interfere with the color reaction. Experiments give consistently 96 to 100 per cent recovery of added crystalline pyridoxine when the adsorption time does not exceed 5 minutes. With an adsorption time of 20 minutes the recoveries were lower, ranging from 80 to 90 per cent. Zeolite, prepared as for use in thiamine analysis, can be used in place of Amberlite IR-4. The use of a synthetic resin of this type has the advantage over the natural clays in that the properties and purity are more uniform. These results are contained in Table II.

TABLE II  
*Recovery of Pyridoxine with Amberlite IR-4 and p-Aminoacetophenone As  
Parts of Process*

Material	Found	Recovery
	$\gamma$	per cent
100 $\gamma$ crystalline pyridoxine hydrochloride.....	98.6	98.6
200 " " " " .....	195.4	97.7
50 " " " " .....	47.9	95.8
100 " pyridoxine + 1 gm. brewers' yeast (75.2 $\gamma$ ).....	172.8	98.7

Experiments with synthetic resin (Amberlite IR-100) show that it adsorbs pyridoxine completely from solution, similar to the action of Super Filtrol, but unfortunately cannot be used in the place of Super Filtrol in this procedure because of the color imparted to the eluate. We find that Amberlite IR-100 also adsorbs riboflavin from acid solutions. Alkaline alcohols other than ethyl alcohol were tried as eluates but certain disadvantages were encountered with each alcohol tried that showed them to be inferior to ethyl alcohol for this purpose. The list included methyl, normal, and isopropyl alcohol.

Scudi *et al.* (5) have shown that boric acid reacts with crystalline pyridoxine to form a complex that prevents its reaction with the chloramide reagent to give the characteristic color phenomena of pyridoxine. We find this to be true with diazotized *p*-aminoacetophenone when crystalline pyridoxine is employed. However, when boric acid is added to extracts of biological materials, interfering side reactions are encountered that prevent its use as a blank in such products. A sample of yeast when

treated according to our regular procedure gave normal values for the pyridoxine content of the yeast and complete recoveries for any added pyridoxine, but when boric acid was used in the blank the values for the yeast were low and erratic and added pyridoxine could not be completely recovered. The strength of the acid used in the hydrolysis and the concentration of boric acid employed in the blank affect the results to a marked extent.

Table III gives typical results obtained when the borate blank was employed on this yeast sample when 0.2 gm. of boric acid was added to 10 ml. of the final alcoholic extract before regular color development.

The chief advantage of the use of *p*-aminoacetophenone over sulfanilic acid lies in the fact that the diazo reaction with pyridoxine takes place at pH 7.0 to 7.3, while the diazo reaction with sulfanilic acid requires a pH of 10 or higher. At this alkaline concentration color is produced

TABLE III  
*Effect of Boric Acid Blank on Acid-Hydrolyzed Extracts*

Material	0.1 N H <sub>2</sub> SO <sub>4</sub>	0.1 N H <sub>2</sub> SO <sub>4</sub>	0.5 N H <sub>2</sub> SO <sub>4</sub>	N H <sub>2</sub> SO <sub>4</sub>
	Without borate blank	With borate blank		
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
1 gm. yeast.....	71.8	16.8	19.9	24.6
1 " " + 100 $\gamma$ pyridoxine.....	169.0	80.4	88.0	76.7

from substances that are not affected at the lower pH value and interfering substances are created that have to be eliminated prior to this treatment. The elimination of the subsequent treatment with alkali, therefore, becomes a distinct advantage to the method.

In color reactions of this type too much emphasis cannot be placed on the cleanliness of the glassware employed. We have observed that the simple reuse of the pipette, without thorough rinsing with distilled water, in the pipetting of the diazo reagent introduces an error that gives rise to a higher value for pyridoxine. This reagent is unstable and produces a chromogen when exposed to the air on a pipette that adds color to the solution on reuse.

The values we obtain for pyridoxine in the materials so far tested are shown to be approximately the same with either of the color reagents employed. The same values are also obtained whether the purification of the extract is carried out with sodium tungstate or by the use of Amberlite. The values by the chemical methods, however, are somewhat higher than the values by the biological method. Yeast that consistently yielded

values of 60 to 65  $\gamma$  per gm. when analyzed by these chemical methods yielded 50 to 55  $\gamma$  per gm. by the biological procedure. We have been unable to establish whether this difference is due to substances other than pyridoxine that produce color that is measured in the process or whether the crystalline pyridoxine used as reference in the biological method is more available to the animals.

#### SUMMARY

1. A new diazo reaction for pyridoxine is described, in which diazotized *p*-aminoacetophenone is used as the color reagent.
2. A synthetic resin, Amberlite IR-4, is found suitable for the removal of interfering compounds in the purification of the extracts for analysis. The use of this resin materially simplifies the purification procedure.
3. The use of a boric acid blank is not applicable when applied to biological materials containing pyridoxine. Added pyridoxine could not be recovered completely and erratically low yields were obtained on biological extracts when this blank was employed.
4. Alkaline methyl alcohol, normal propyl alcohol, and isopropyl alcohol were tried as eluates but proved to be inferior to ethyl alcohol for this purpose. Alkaline alcohol does not stabilize the color complex formed with diazotized *p*-aminoacetophenone and pyridoxine as it does with sulfanilic acid.

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# AMINO ACID INHIBITION OF COPPER PROTEINATE FORMATION

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In an investigation of the applicability of the copper sulfate method (1) for protein determination in sera of different species, three therapeutic antipneumococcus rabbit sera, which were about 5 years old, failed to form the typical copper proteinate sac. However, the falling drop (2) and the refractometric (3) methods indicated protein concentrations in these three sera to be about the same as in other sera from similar sources. The failure of these sera (one each of types 1, 8, and 17) to form the copper membrane was found to be directly related to the presence in the serum of an excess of free amino acids. We have been able to induce this condition in normal serum by the addition of amino acids or by enzymatic processes. This failure to form the copper proteinate sac is likely to be encountered only rarely (in the present experience, three in 120 instances) and under abnormal conditions; it should therefore not greatly affect practical applications of the method.

## EXPERIMENTAL

In search of an explanation of the anomalous behavior of the three sera, a study was made of the routine methods in the preparation of such sera for human use. The usual treatment of antipneumococcus rabbit sera in these laboratories includes heating at 56°, the addition of two preservatives, "merthiolate" to give a final concentration of 1:20,000 and phenol in ether to give a final concentration of 1:500, and adsorption with kaolin. These procedures were found to have no apparent effect on the precipitability of serum protein by copper sulfate.

*Effect of Amino Acids*—Bourdillon (4) has reported that the products of protein hydrolysis may interfere with precipitation of the protein upon the addition of heavy metal salts. In order to reduce the concentration of amino acids that might be present, a sample of one of the three sera in question, type 8, was dialyzed overnight against tap water in a collodion bag. A drop of the residue in the copper sulfate solution<sup>1</sup> formed a proteinate in characteristic manner. Addition of 2.5 mg. of glycine per ml. to some of the

<sup>1</sup> A 4 per cent  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (sp. gr. approximately 1.025) is convenient for this purpose.

residue resulted in inhibition of copper proteinate precipitation, as before dialysis. Approximately 0.2 per cent of glycine added to normal rabbit serum was found to produce a like effect. Similar results were obtained with equimolar concentrations (about 38 mg. of  $\alpha$ -amino nitrogen per 100 ml.) of *l*-histidine monohydrochloride, *dl*-alanine, *dl*-valine, and *l*-proline.

*Neutralization of Inhibition*—Glycine added to samples of human serum induced a similar inhibition. Subsequent addition to the serum of normal NaOH to make the pH approximately 8.6, or of 0.4 per cent of formalin, followed by incubation, as in the detoxification of diphtheria toxin (5), neutralized the effect of the amino acid, and proteinate sacs were again formed when drops of serum were added to copper sulfate solution. The formalin treatment was satisfactorily employed on a small portion of the type 8 serum used above and a determination of protein by the copper sulfate method indicated the presence of 8.65 gm. per 100 ml. This compared favorably with the values of 8.59 and 8.33 gm. per 100 ml. obtained respectively in tests of the original serum by the falling drop and by the refractometric methods. Although in the different methods for protein determination conversion factors established for species other than rabbits are employed, the various protein figures agree fairly well. In the copper sulfate test for protein determination the factor 376 for normal human serum (1) was employed in the place of 347.9 used by Weech, Reeves, and Goettsch (6).

*Concentration of Free Amino Acids Responsible for Inhibition of Precipitation*—Gasometric determinations of free amino acids in samples of normal and immune rabbit sera were made by the ninhydrin-carbon dioxide method (7). We are indebted to Dr. W. R. Thompson for this work. The anti-pneumococcus rabbit sera from which the protein previously had been shown to be non-precipitable by copper sulfate contained from 45.86 to 104.1 mg. of  $\alpha$ -amino nitrogen per 100 ml., whereas four immune sera that formed typical copper proteinate contained from 8.34 to 22.27 mg. per 100 ml. In a pool of normal rabbit sera, there were 6.60 mg. per 100 ml.

Hamilton and Van Slyke (7) point out that for accurate determinations of the  $\alpha$ -amino nitrogen in the circulating plasma, blood samples must be handled with care to avoid hemolysis or injury of cells. Because the cells may contain a concentration of  $\alpha$ -amino nitrogen twice as great as that of plasma, a positive error may result through the diffusion of amino acids from the cells into the plasma. Probable evidence of such an effect was observed in results of gasometric determinations on two individual normal rabbit sera. One serum, notably hemolyzed, gave a value of 7.38, whereas a second serum, of normal appearance, gave a value of 5.64 mg. of  $\alpha$ -amino nitrogen per 100 ml.

It is important to note that, according to MacFadyen (8), "When either

whole blood or separated plasma clots, the amino acid content of the serum formed is usually 10 to 40 per cent greater than that of the plasma." Analyses of serum cannot be considered, therefore, an accurate measure of the amino acid content of the circulating plasma.

TABLE I  
*Results of Tests with Normal and Immune Rabbit Sera*

Rabbit serum	Protein determination*		pH	Viscosity (water = 1) at 25°	$\alpha$ -Amino N†  mg. per 100 ml.	Remarks
	Copper sulfate method	Refracto- metric method				
Antipneumococcus, R-8-63	No proteinate formed	8.33	7.4	1.64	104.1	
Antipneumococcus, R-1-19	Only sugges- tion of ppt.	7.74		1.62	55.18	
Antipneumococcus, R-17-20	Partial, weak ppt.	8.60		1.76	45.86‡	
Antipneumococcus, R-32-11	7.63	7.64		1.70	22.27	
Antipneumococcus, R-14-46	7.71	7.72		1.75	18.36	
Antipneumococcus, R-4-12	8.01	8.05		1.83	16.04	
Antipneumococcus, R-14-28	7.29	7.22		1.73	8.34	
Normal, R-H920	6.51	6.52			7.38	Serum hemo- lyzed
" R-H203	5.73	5.65			5.64	Serum non- hemolyzed
" (1944 bleedings)	5.45-6.99	5.09-6.57			6.60	
Normal pools	6.02-6.88	6.05-6.49	7.6§	1.21-1.41		Sera obtained 1939-43

\* Protein content recorded in gm. per 100 ml.

† Ninhydrin-carbon dioxide method; 0.1 mg. per 100 ml. subtracted as a correction for urea present, assuming the normal range, according to Hamilton and Van Slyke (7).

‡ A 0.2 per cent solution of glycine would have approximately 38 mg. per 100 ml. of  $\alpha$ -amino nitrogen.

§ Serum not stored in resistant glass vial.

Results of tests of various rabbit sera are given in Table I.

*Effect of Enzyme Action*—Data obtained in viscosity tests of rabbit sera, a method employed to follow the course of enzyme activity, suggested that we were dealing with products of enzyme action. In this instance proteo-



lytic enzymes that yield amino acids as end-products of protein digestion were indicated. In our tests with a commercial preparation of trypsin (Difco 1:250), samples of normal rabbit serum incubated with a small amount of enzyme overnight at approximately 39° were altered so as to inhibit copper proteinate formation partially or wholly.

The staphylococcus was used in a study of the degree to which free amino acid might be produced in serum by the proteolytic activity of an enzyme of a microorganism. We chose a culture of *Staphylococcus aureus* in preference to *Staphylococcus albus* because of its possible greater enzyme activity. The inoculum was suspended in saline and added to several normal sera. After approximately 2 weeks of incubation at 35°, normal rabbit and normal guinea pig sera failed to form a copper proteinate.

#### DISCUSSION

Bourdillon has observed considerable hydrolytic changes in several 9- to 12-year old diphtheria antitoxic horse sera and has considered the possibility that such changes may be caused by some native enzyme in the original serum. It is possible that the hydrolytic changes which had occurred in our therapeutic rabbit sera were similar to those noted in the antitoxic horse sera.

#### SUMMARY

Three therapeutic rabbit serum pools which were about 5 years old at the time these tests were made failed to form the copper membrane. Other pools of approximately the same age maintained under the same conditions reacted normally in the copper solution. These sera, which had been distributed for therapeutic use, had been maintained in the laboratory before distribution, and, presumably, later in the district supply stations at temperatures under 10°. The tests were made after the sera had been returned at their date of expiration.

Three rabbit sera which did not form the copper proteinate contained from 45.86 to 104.1 mg. of  $\alpha$ -amino nitrogen per 100 ml. Free amino acids in serum, equivalent to about 46 mg. of  $\alpha$ -amino nitrogen per 100 ml., have been found to inhibit formation of copper proteinate sacs when the serum is dropped into a solution of copper sulfate. A similar result was obtained by incubation of sera with trypsin or with a culture suspension of *Staphylococcus aureus*.

The inhibitory influence of the free amino acids can be overcome by the addition of sufficient alkali or by treatment with formalin.

The amino acid inhibition of proteinate formation in the presence of copper sulfate solution affords a simplified test of tryptic digestion in serum and perhaps in other substrates.

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## AN ACCELERATOR OF CATALASE ACTIVITY

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Recently, it was shown that yeast extracts accelerated the oxidation of various substrates by hydrogen peroxide in the presence of the enzyme peroxidase (1). The nature of this stimulation is not known; however, two observations led us to the view that the active principles were iron complexes whose action was bound in some way to that of the enzyme.

The first observation was the very slight but definite peroxidase activity of the extracts. Iron complexes (2) and hemins and metallic iron in various physical states (3, 4) have been shown to possess this ability to oxidize phenols. The yeast extracts had very little peroxidase activity alone, but in combination with the enzyme preparation gave increased oxidation.

The second observation was the ability of the extracts to antagonize cyanide and azide inhibition of the enzyme, an action which seemed to be closely related to the ability to accelerate the peroxidase in that higher concentrations of the inhibitors affected the stimulated action as well as normal peroxidase action. It seemed to us therefore that the yeast extract was acting in a manner similar to the hemin portion of the enzyme.

It may be mentioned that these yeast extracts have for some time been known to stimulate the oxygen uptake of such tissues as skin and liver and of some microorganisms, including bakers' yeast (5). Here too, the stimulatory action was shown to be concerned with the iron portion of the respiratory chain, since cyanide and azide, which are known to attack specifically the cytochrome oxidase, could be completely antagonized, whereas amyl alcohol and urethane inhibition could not (6).<sup>1</sup>

In the present paper it is demonstrated that the yeast extract accelerates hydrogen peroxide splitting by the enzyme catalase, and an attempt has been made to study the nature of this action.

### EXPERIMENTAL

Since we were interested in relative activities alone, all determinations were made at 24° in phosphate buffer (pH 6.8) by a procedure similar to that of von Euler and Josephson (7). After an equilibrium period of 10 minutes, which allowed the solutions to come to temperature, 35 ml. of

<sup>1</sup> Kreke, C. W., and Suter, M. St. A., to be published.

The yeast extract corresponded to Sample A prepared as described previously (5). The extract was adjusted to pH 6.8 and sterilized. Dilutions were made in the phosphate buffer.

In Fig. 1 is shown the effect of yeast extract (1 mg. per ml.) on the  $H_2O_2$  decomposition by catalase. The stimulation (Curve II) amounted to approximately 63 per cent over the control (Curve I) at the 10 minute period. (Concentrations around 0.5 to 1.0 mg. per ml. seem to be an optimum. Concentrations of 5 mg. per ml. or higher produce depression.)

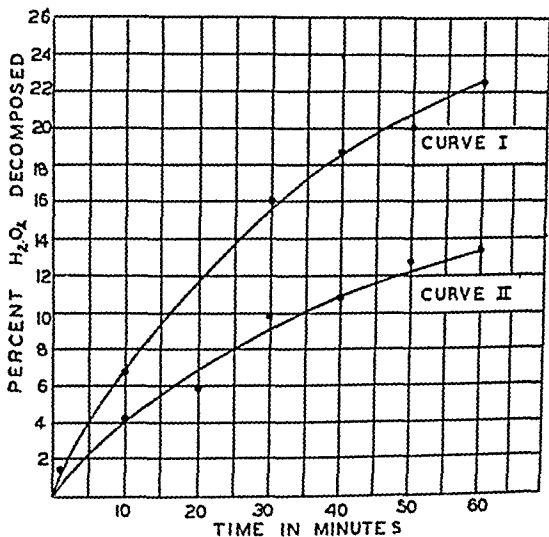


FIG. 3. Effect of yeast extract on hemin decomposition of  $H_2O_2$ . Curve I, hemin control; Curve II, hemin control plus yeast extract, 1 mg. per ml.

The acceleration is similar to that produced by the presence of additional enzyme, since there is an initial increased rate with a falling off as the  $H_2O_2$  is used up.

It is interesting, however, that the enzyme-like action of the extract is not expressed by itself but only in the presence of catalase. That the yeast extract itself is not capable of decomposing  $H_2O_2$  is shown in Table I. 4.95 ml. of 0.01 N  $KMnO_4$  correspond to zero decomposition. Concentrations from 0.5 to 5 mg. per ml. of extract were tested with the same results. If the active principles were iron complexes, as we suggested in the case of peroxidase, one would expect a slight but definite catalase activity. It is possible that the active principles are at too low a concentration here to show any such action during the 40 minute experimental period.

The ability of the yeast extract to offset cyanide inhibition of the enzyme

is shown in Fig. 2. Curve II shows the depression produced by  $4 \times 10^{-6}$  M KCN from the normal control value (Curve I). Curve III shows the ability of the extract (1 mg. per ml.) to overcome the depression at least in part. At times it was possible to bring about a complete antagonism and produce a slight stimulation with smaller concentrations of inhibitor. With higher concentrations of inhibitor less effect was noticeable with the extract, probably due to a reaction between the inhibitor and the extract.

That the extract requires the enzyme for its action and not the iron prosthetic group alone is shown in Fig. 3 in which hemin decomposition of  $H_2O_2$  (Curve I) is depressed by 1 mg. per ml. of extract (Curve II). The concentration of hemin used here was  $2.5 \times 10^{-4}$  mg. per ml., amounting to several million times the hemin present in the catalase experiments. 0.5 mg. per ml. of the extract also gave depression. Other experiments with higher concentrations of extract gave increasing amounts of depression,

TABLE II

*Effect of Various Yeast Extract Concentrations on Hemin Decomposition of  $H_2O_2$*

Concentration yeast extract mg. per ml.	Per cent depression over control in 10 min.
0.5	26.6
1.0	39.0
3.0	50.0
5.0	100.0

as shown in Table II. This complete reversal in action for enzyme and hemin is indicative of a specific function of the extract which involves the entire enzyme.

#### DISCUSSION

A large amount of literature has accumulated which shows that iron in various degrees of complexity possesses oxidase, peroxidase, and catalase abilities to a small extent as compared with the specific enzymes. For instance, 1 hemin molecule is able to decompose  $10^{-2}$  molecule of  $H_2O_2$  per second compared with 1 molecule of catalase which decomposes  $10^{+5}$  molecule of  $H_2O_2$ . 1 atom of inorganic ferric iron splits only  $10^{-5}$  molecule of  $H_2O_2$  per second. This is of interest in view of the fact that the yeast extract could increase the catalase activity approximately 63 per cent in 10 minutes when present at a concentration of 1 mg. per ml.

Recent work in our laboratories has shown that ferric citrate in a concentration of 1:100,000 had no catalase activity during the experimental period, but accelerated liver catalase as much as 37 per cent. We suggest that iron complexes may be responsible for the results with the extract.

Whether constituents of the yeast extract can combine with the proteins of the liver extract to function as prosthetic groups or whether they act by reoxidizing the reduced enzyme will be the subject of future work.

#### SUMMARY

In this work, an alcoholic yeast extract has been shown to accelerate the action of catalase when present in a concentration of 0.5 to 1.0 mg. per ml. This effect is due to a direct acceleration of the enzyme, since the extract had no effect by itself.

When tested on hemin decomposition of  $H_2O_2$ , no acceleration was obtained. It was suggested that the yeast extracts contained iron complexes which may act by keeping the enzyme in the oxidized form.

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# CARBONATE CONTENT OF BONE IN RELATION TO THE COMPOSITION OF BLOOD AND DIET\*

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The composition of the inorganic portion of bone is not constant. This composition has been represented by the formula  $\text{CaCO}_3 \cdot n\text{Ca}_3(\text{PO}_4)_2$  (1-3). The greatest variations occur in the percentage of carbonate. These changes have been found to occur with age (4, 5), rickets (4, 6), acidosis (7-12), alkalosis (12), diet (4, 8, 12, 13), and osteopetrosis (14).

In the studies made by previous investigators, no serious attempt was made to interrelate the changes in bone to changes in blood, since these investigations sought other relationships. However, a distinct trend is evident if the data on blood changes in these conditions are collected and examined. It has been pointed out by Howland *et al.* (6) and Kramer *et al.* (14) that the changes in the composition of bone may be a reflection of the composition of the blood. As Kramer, Yuska, and Steiner (14) point out, "The relationship of the composition of bone to that of blood deserves further investigation," and "This relationship of bone composition to blood composition in infants is a wholly unexplored field." These statements may be expanded to include the whole field of blood and bone relationships.

*In vitro* experiments on the preparation of solid phases resembling those present in bone have shown that an increase of the carbonate in the precipitate is a function of the carbonate to phosphate ratio of the liquid phase containing calcium, phosphate, and carbonate ions. This fact is demonstrated in data given by Logan and Taylor (15) and by Howland, Marriott, and Kramer (6). If we accept the premise that bone has an apatite structure (as has been concluded from x-ray evidence), then the composition of the solid should reflect the composition of the liquid phase from which it forms, since apatite structures have been shown to form a continuous series of solid solutions (Eisenberger *et al.* (16)).

In the experiments reported here, evidence is presented that a relationship apparently exists between the carbonate content of bone and the composition of serum. The composition of the serum was altered by dietary

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means. The knowledge of the factors that influence the composition of the bone is of theoretical and practical importance, since further studies may reveal some relationship between structural, mechanical, and other properties (such as resistance to disease) and composition.

### *Preliminary Experiments*

In the first, as well as in all subsequent, experiments young rats 23 days old were used. The results indicated that as the inorganic phosphorus of the serum increased the carbonate of the bone decreased. Rickets was produced by both low calcium-high phosphorus and high calcium-low phosphorus diets. However, only in the case of rickets produced on a high calcium-low phosphorus diet was there an increase in bone carbonate. In

TABLE I

*Composition of Blood and Bone in Relation to Diet; Preliminary Experiments (Mean Values)*

Dietary group	No. of rats	Change in weight	Serum		Femora, dry, fat-free					
			Ca	P	Weight of bone	CO <sub>2</sub>		Ca		CO <sub>2</sub> : Ca
			gm.	mg. per cent	mg.	mm X 1000	per cent	mm X 1000	per cent	mole ratio
Diet A, low Ca-low P.....	13	+22	5.0	7.4	74.2	17.6	1.42	169	9.14	0.104
Diet B, high Ca-low P.....	14	+32	10.1	3.3	89.9	28.9	1.93	223	9.92	0.130
Diet C, low Ca-high P.....	8	+13	5.3	8.6	58.9	15.5	1.58	146	9.93	0.106

the case of rickets produced on a low calcium-high phosphorus diet, there was a tendency towards decreased carbonate in the bone. In rickets due to high phosphate diet the serum inorganic phosphorus was normal, while the serum calcium was low, whereas in rickets due to low phosphate diet the serum calcium was high, while the serum inorganic phosphorus was low. There were indications that the composition of the blood followed that of the diet, as has been previously reported (17-19). The data for these experiments are not given, since they mainly served in the development of the analytical procedures (20). The diets used in these experiments were lower in phosphorus content than the later diets, which are described in this paper. Because of this, more severe rickets was produced in our earlier experiments. Data on these diets are given below.

Table I summarizes the values obtained in our later experiments which were still of a preliminary nature, but since the new micromethods were employed, these data were considered valid.

The diets were similar to those in Table II, except that the phosphorus levels were 0.05 per cent lower. It may be observed in Table I that the addition of calcium to Diet A caused an increased  $\text{CO}_3\text{:Ca}$  ratio in the bone when compared to results on the low calcium-low phosphorus group, while the high phosphorus-low calcium group did not show a significant change in the  $\text{CO}_3\text{:Ca}$  ratio of the bone. There was an increase in the calcium content of the bone in the group on Diet B, but the increase in carbonate was greater than that of calcium. In the group on Diet C there was a decrease in both calcium and carbonate of the bone compared with Diet A. The serum inorganic phosphorus was decreased in the group on Diet B and was slightly increased with Diet C. The serum calcium increased in the group on Diet B and remained unchanged with Diet C when these groups

TABLE II  
*Composition of Experimental Diets*

Diet	Constituents		Ca	P
		<i>parts</i>	<i>per cent</i>	<i>per cent</i>
A, low Ca-low P (basal)	Yellow corn-meal	70	0.030	0.32
	Wheat gluten	16		
	Brewers' yeast	10		
	NaCl	1		
B, high Ca-low P	Basal diet	100	1.13	0.317
	$\text{CaCO}_3$	3		
C, low Ca-high P	Basal diet	100	0.029	0.905
	$\text{Na}_2\text{HPO}_4$	2.75		

were compared with those on Diet A. There appeared to be a rough reciprocal relationship between serum inorganic phosphorus and bone carbonate.

### *Final Experiments*

These were designed to supply more complete data on the changes observed in the exploratory work. Analyses of serum  $\text{CO}_2$  were performed to determine whether any relationship existed between serum carbonate and bone carbonate. In addition, the experiments were extended to include the influence of vitamin D.

Three diets were used in these experiments. The basal diet contained traces of calcium (0.03 per cent) and suboptimum amounts of phosphorus (0.32 per cent). This diet without other additions will be referred to in this paper as a low calcium-low phosphorus diet (Diet A). However, the Ca:P ratio of this diet is low (less than 0.1). It is therefore actually a low

formula  $\text{CaCO}_3 \cdot n\text{Ca}_3(\text{PO}_4)_2$ , which represents the inorganic composition of bone with respect to calcium, phosphorus, and carbonate.

$$\frac{\text{Calcium combined with CO}_2}{\text{Calcium combined with PO}_4} = \frac{\text{mm CO}_2}{\text{mm Ca} - \text{mm CO}_2}$$

The mean results were evaluated by the statistical methods of Fisher (26) as applied to small samples. *P* represents the frequency with which the difference between two means may be due to chance alone. When *P* = 0.05 or less, then the difference between the means may be considered statistically significant.

*Weight Changes*—The experimental animals were normal in appearance and behavior. The weight changes (growth), including the initial and final weights, are indicated in Table III. The first three *P* values appearing in Table III indicate the statistical significance of differences in growth resulting from the influence of dietary calcium and phosphorus in the absence of vitamin D. In Group A, the growth was 23.7 gm., while on the addition of calcium to Diet A (Diet B), the growth increased to 45.0 gm., which is only slightly below the normal weight for rats of this age. (The normal growth of rats in our colony is about 53 gm. for a similar period.) A decreased growth of 17.2 gm. resulted from the addition of phosphate to Diet A (Diet C). The differences between Group B and Groups A and C were significant. In contrast, the differences between Groups A and C were not significant. In the presence of vitamin D, dietary calcium and phosphorus had a negligible influence on growth. There was better growth in all groups with vitamin D, but the changes due to dietary calcium and phosphorus are not statistically significant (see *P* Values 4 to 6).

The influence of vitamin D on growth is evident from Values 7 to 9 of *P*, obtained by the statistical analysis of the resulting growth changes. There was a significant increase in growth in the low calcium-low phosphorus and the low calcium-high phosphorus groups. In the high calcium-low phosphorus group, in which growth was greatest, the addition of vitamin D had no significant influence.

The rickets produced in the absence of vitamin D was mild in character, since the phosphate level of the diet approached the optimum level quite closely and the Ca:P ratio was not extreme (18, 26).

#### *Composition of Bone and Blood Serum*

*Influence of Dietary Calcium, Phosphorus, and Vitamin D*—The results obtained from the analyses of the bones and blood sera are presented in Table IV and the statistical evaluation of the data is given in Table V.

*Total Carbonate and Calcium in Bone*—The highest amounts of carbonate

and calcium were found in Group B, the differences between this and Groups A and C being significant both with and without vitamin D (Table V, Lines 1, 3, 4, 6). In contrast to this, the differences between Groups A and C were not significant (Table V, Lines 2, 5). In comparing the vitamin-fed members of each group to those not fed this supplement, the influence of the antirachitic vitamin was manifested by (a) significant increase in Group B of carbonate and calcium, (b) significant increase of only calcium in Group A, and (c) no significant changes in Group C.

*Percentage of Carbonate and Calcium in Bone*—In the absence of vitamin D Group B has a distinctly higher percentage of carbonate than the other two groups, but the difference in the percentage of calcium is of border line significance statistically (Table V, Lines 1 to 3). Thus the relative amount

TABLE IV  
*Bone Carbonate and Calcium in Relation to Composition of Blood and Diet*  
(Mean Values)

Dietary group	Blood serum			Weight of bone	Bone, dry, fat-free femora				
	Ca	P	CO <sub>2</sub>		Ca	CO <sub>2</sub>	CO <sub>2</sub> / Ca	Ca	CO <sub>2</sub>
	mg. per cent	mg. per cent	vol. per cent		mm × 1000	mm × 1000	molar ratio × 100	per cent	per cent
A.....	6.3	8.9	58.8	74.5	189.3	20.96	11.1	10.39	1.74
B.....	11.8	4.2	59.0	93.4	297.9	45.25	15.3	13.02	2.82
C.....	6.0	9.4	56.9	69.4	196.4	23.28	11.9	11.45	2.04
A + vitamin D.....	8.7	8.9	54.7	81.6	235.4	23.80	10.2	11.80	1.80
B + " ".....	13.3	6.8	57.1	129.8	546.3	75.77	13.9	17.00	3.53
C + " ".....	8.8	9.3	52.6	77.4	242.3	23.91	9.9	12.50	1.85
Reference.....	11.1	10.3	51.6	40.6	153.7	13.89	9.1	15.26	2.08

of carbonate deposition proceeded much faster than that of the calcium. There are no statistically significant differences between Groups A and C with regard to the percentage values of carbonate and calcium (Table V, Line 2).

In the presence of vitamin D, Group B had a significantly higher percentage of carbonate as well as calcium compared to the other two groups. There were again no statistically significant differences between Groups A and C (Table V, Lines 4 to 6).

Vitamin D increased the percentage of calcium in all groups, and carbonate in Groups B and A, but only that in Group B was statistically significant (Table V, Lines 7 to 9).

*CO<sub>2</sub>:Ca Ratio in Bone*—The highest CO<sub>2</sub>:Ca ratios were found in Group B, both with and without vitamin D. The differences between Group B

TABLE V  
Statistical Evaluation (Cf. (95)) of Mean Values Presented in Table I

The results are expressed as *P* values.

Line No.	Dietary groups	L blood serum			Bone, dry, fat-free femora					
		Ca	P	CO <sub>2</sub>	Ca	CO <sub>2</sub>	CO <sub>2</sub> :Ca	C <sub>a</sub>	CO <sub>2</sub>	per cent
		mg. per cent	mg. per cent	vol. per cent	mM × 1000	mM × 1000	molar ratio × 100	per cent		per cent
1	A vs. B	<10 <sup>-10</sup>	<10 <sup>-10</sup>	1.00	<10 <sup>-7</sup>	<10 <sup>-10</sup>	<10 <sup>-3</sup>	0.047	<10 <sup>-4</sup>	<10 <sup>-4</sup>
2	" " C	0.61	0.41	0.73	0.71	0.15	0.19	0.59	0.09	0.09
3	B "	<10 <sup>-10</sup>	<10 <sup>-10</sup>	0.66	<10 <sup>-5</sup>	<10 <sup>-10</sup>	<10 <sup>-8</sup>	0.24	0.018	0.018
4	A + vitamin D vs. B + vitamin D	<10 <sup>-10</sup>	<10 <sup>-4</sup>	0.42	<10 <sup>-4</sup>	<10 <sup>-10</sup>	<10 <sup>-9</sup>	<10 <sup>-9</sup>	<10 <sup>-10</sup>	<10 <sup>-10</sup>
5	A + " " C + " "	0.80	0.51	0.48	0.78	0.88	0.39	0.50	0.78	0.78
6	B + " " " + " "	<10 <sup>-10</sup>	<10 <sup>-5</sup>	0.11	<10 <sup>-4</sup>	<10 <sup>-10</sup>	<10 <sup>-9</sup>	<10 <sup>-9</sup>	<10 <sup>-10</sup>	<10 <sup>-10</sup>
7	A vs. A + vitamin D	<10 <sup>-8</sup>	1.00	0.37	0.04	0.33	0.05	0.38	0.73	0.73
8	B " B + " "	<10 <sup>-8</sup>	<10 <sup>-7</sup>	0.62	0.015	<10 <sup>-6</sup>	0.04	<10 <sup>-5</sup>	0.017	0.017
9	C " C + " "	<10 <sup>-8</sup>	0.87	0.252	0.051	0.76	<10 <sup>-5</sup>	0.35	0.28	0.28
10	Reference vs. A	Sig.	<10 <sup>-4</sup>	0.33	0.080	<10 <sup>-5</sup>	Sig.	Sig.	0.66	0.66
11	" " B	0.019	Sig.	0.29	Sig.	Sig.	"	0.04	0.017	0.017
12	" " C	Sig.	0.14	0.37	0.075	<10 <sup>-9</sup>	"	Sig.	0.76	0.76
13	" " A + vitamin D	"	Sig.	0.31	<10 <sup>-3</sup>	Sig.	0.01	"	0.07	0.07
14	" " B + " "	<10 <sup>-10</sup>	"	0.28	Sig.	"	Sig.	<10 <sup>-3</sup>	<10 <sup>-10</sup>	<10 <sup>-10</sup>
15	" " C + " "	<10 <sup>-10</sup>	"	0.79	"	"	0.04	<10 <sup>-4</sup>	0.10	0.10

*P* = the probability that the difference between the two means is due to chance.

Sig. = the difference between two means is significant. This method was employed only in comparing the reference group against the others, Lines 9 to 15. These values were not calculated because, by inspection of the differences between the means and the squares of the standard deviation, *P* would be lower than the next lowest *P*.

and the other two were significant (Table V, Lines 1, 3, 4, 6). In contrast, the differences between Groups A and C were not significant (Table V, Lines 2, 4). Vitamin D caused a decrease of the  $\text{CO}_3:\text{Ca}$  ratio in all three groups (Table V, Lines 7 to 9).

*Calculated  $\text{PO}_4:\text{Ca}$  Ratio in Bone*—The  $\text{PO}_4$  content of the bone may be calculated by

$$[\text{Calculated } \text{PO}_4, \text{ mm}] = [\text{Ca, mm}] - [\text{CO}_2 \text{ in mm}]$$

The above equation is true only if we accept the inorganic composition of bone as  $[\text{Ca}_3(\text{PO}_4)_2]_n \cdot \text{CaCO}_3$ . There are actually slight deviations from this formula; nevertheless, the calculated  $\text{PO}_4$  can give a close approximation of the real values and indicate trends.

The trend in the  $\text{PO}_4:\text{Ca}$  ratios is the exact opposite from that found for the  $\text{CO}_3:\text{Ca}$  ratios. It may be worth while to note that in both of the high calcium-low phosphorus groups (Group B) the absolute amount of phosphate was higher than in the other two. However, the relative increase in calcium tied up as the carbonate was still greater and thus the  $\text{PO}_4:\text{Ca}$  ratio was lower in these two groups. The  $\text{PO}_4:\text{Ca}$  ratio was higher in the vitamin-fed groups than in the corresponding groups without the supplement. Here the total amount of all constituents increased but the relative amount of calcium combined as carbonate increased more than that combined as the phosphate.

*Composition of Bone in Relation to Blood Serum*—The serum  $\text{CO}_2:\text{P}$  ratio was highest in Group B, in which the bone  $\text{CO}_3:\text{Ca}$  ratio was also the highest (Table V, Lines 1, 3, 4, 6). The differences in the serum  $\text{CO}_2:\text{P}$  in Groups A and C are small and not significant. Similarly, the differences between bone  $\text{CO}_3:\text{Ca}$  ratios in Groups A and C are also without significance (Table V, Lines 2, 5).

Under the influence of vitamin D the serum  $\text{CO}_2:\text{P}$  ratio dropped in all groups. Concurrently, a statistically significant drop took place in the  $\text{CO}_3:\text{Ca}$  ratio (Table V, Lines 7 to 9). Here it must be pointed out that the serum  $\text{CO}_2$  dropped in all groups under the influence of vitamin D. These differences individually are not statistically significant (Table V, Lines 7 to 9). However, when all of the vitamin D-fed groups are compared with the non-vitamin groups by the pair method, the trend towards decreased serum  $\text{CO}_2$  values is statistically significant. This point bears further investigation.

*Comparison of Results against Reference Group*—The last line of Table IV shows the results obtained in the reference group which represents the conditions existing at the beginning of the experiment. In all groups, there is an evident increase in the weight of the bone and in the calcium and carbonate content. The degree of mineralization, as shown by the values

for the percentage of calcium, however, decreased in all groups with the exception of Group B fed vitamin D, in which it was higher. All these changes mentioned were statistically significant. This may be interpreted as indicating that the organic portion of the bone increased faster than the inorganic portion. The degree of carbonate deposition, as shown by the percentage of carbonate in the bone, decreased in Groups A and C, but not in Group B, in which an increase is evident. The  $\text{CO}_3:\text{Ca}$  ratio of the bone increased in all groups when compared to the reference group. Thus, in an indirect way, the influence of age on the carbonate content of the bone is expressed.

Changes in the ratio of bone  $\text{CO}_3:\text{Ca}$  appear to follow the serum  $\text{CO}_2:\text{P}$  ratios. The bones of animals fed high calcium diets showed a significantly higher  $\text{CO}_3:\text{Ca}$  ratio than the other two groups. The influence of vitamin D was manifested in the lower  $\text{CO}_3:\text{Ca}$  ratio in the bone on corresponding diets and lower  $\text{CO}_2:\text{P}$  ratios in the blood. If all seven groups are arranged in the order of serum  $\text{CO}_2:\text{P}$  ratios, Group C without vitamin D omitted, this order will also be followed by the values for bone  $\text{CO}_3:\text{Ca}$  of the six remaining groups. Group C falls out of line, but by a scant difference. Studies on the complete composition of bone (which will soon be submitted for publication) show that the residual  $\text{Ca}:\text{P}$  ratio in the bones of this group differs from the value usually found.

The density of the bones is in general interrelated to the serum  $\text{Ca} \times \text{P}$  product in this series, with but one exception. In Group B without vitamin D, the serum  $\text{Ca} \times \text{P}$  product is the lowest of all groups. Despite this fact, however, the density of the bones in this group is third highest in the series. Whether the serum  $\text{Ca} \times \text{CO}_2$  product of this group, which ranges second highest in the series of these products, is influential with regard to the degree of calcification in this group cannot be stated. *In vitro* experiments (15) indicate that at least the deposition of carbonate in bone is influenced by this product, even though the solubility product  $[\text{Ca}^{++}] \times [\text{CO}_3^{--}]$  is not reached. Though this fact may be only coincidental, it may be noted that there is a close correlation between the serum  $\text{Ca} \times \text{CO}_2$  product and the calcium and carbonate content of the bone. What bearing this fact may have on the amount of calcium deposited cannot be stated, especially as the experiments of Bethke *et al.* (17) show an opposite trend when values for serum calcium and bone ash are compared. More evidence for a possible relationship between the serum  $\text{Ca} \times \text{CO}_2$  product and the carbonate content of bone should be sought.

#### DISCUSSION

It is evident from our experiments that the calcium and phosphorus content of the diet as well as vitamin D influences the  $\text{CO}_3:\text{Ca}$  ratio of bone.

The high  $\text{CO}_3\text{:Ca}$  ratio in bone was associated with high calcium-low phosphorus diets. A lowered  $\text{CO}_3\text{:Ca}$  ratio was obtained on both the low calcium diets (A and C). A less predominating but statistically significant decrease in bone  $\text{CO}_3\text{:Ca}$  ratio occurred under the influence of vitamin D, which increased the density of the bones in all three dietary groups. Neither rickets *per se* nor low bone density was found to be the predominating factor in bone  $\text{CO}_3\text{:Ca}$  ratio. Rickets produced by low calcium-high phosphorus diets showed low  $\text{CO}_3\text{:Ca}$  ratios in the bone, as compared with the rickets produced in a high calcium-low phosphorus diet in which the  $\text{CO}_3\text{:Ca}$  ratio in the bone was higher. Vitamin D was unable to counteract completely the effect of the rather extreme dietary  $\text{Ca:P}$  ratios on bone  $\text{CO}_3\text{:Ca}$  ratio.

The influence of diet on bone may be explained by its effect on the major inorganic components of bone present in blood, namely, calcium, phosphorus, and  $\text{CO}_2$  (carbonate). As has been previously noted (17-19, 27), the serum calcium and inorganic phosphorus levels reflect the dietary calcium and phosphorus content. On a high calcium-low phosphorus diet, the serum calcium is normal, while the inorganic phosphorus is low. On a low calcium-high phosphorus diet, the serum calcium is low, while the inorganic phosphorus is normal. Vitamin D tends to raise the low member of the pair and in general the more disproportionate the dietary  $\text{Ca:P}$  ratio, the less effective is the vitamin D (17, 19). No serious study of the influence of dietary calcium and phosphorus on the  $\text{CO}_2$  content of serum (which expresses the carbonate content of the blood) is presented in the literature. In these studies, dietary calcium and phosphorus had no detectable influence on blood  $\text{CO}_2$ . Vitamin D caused a lowering of the carbonate in each group. This lowering had no statistical significance in any given dietary group. However, the trend towards decreased serum  $\text{CO}_2$  levels in all three groups is statistically significant. More data of this type are necessary for final conclusions.

It is known that bone is not a precipitate of exact composition. Its structure may be represented by the general formula  $\text{CaCO}_3 \cdot n\text{Ca}_3(\text{PO}_4)_2$ , in which approximately 6 per cent of the calcium may be substituted by other bases (28). X-ray evidence shows that it has an apatite lattice. Apatites in general are considered solid solutions whose composition reflects that of the supernatant fluid (16). Thus, with a given calcium concentration, the carbonate content of the solid phase should be a reflection of the  $\text{CO}_3\text{:PO}_4$  ratio of the liquid phase, from which it follows that the  $\text{CO}_3\text{:Ca}$  ratio of the solid will also be a reflection of the  $\text{CO}_3\text{:PO}_4$  ratio of the liquid phase.

On examining the data from the *in vitro* experiments of Howland, Marriott, and Kramer (6), it may be seen that the  $\text{CO}_3\text{:Ca}$  ratio of the solid formed was a reflection of the  $\text{CO}_3\text{:PO}_4$  ratio of the liquid phase.



This fact is also evident in the data of Logan and Taylor (15, 29). The explanation Logan offers is that carbonate is adsorbed on the crystal lattice of  $\text{Ca}_3(\text{PO}_4)_2$  and that the adsorption is proportional to the  $[\text{Ca}^{++}] \times [\text{CO}_3^{--}]$  product in the supernatant liquid. He offers as support for his concentration the fact that proportionately more carbonate than phosphate can be removed by weak acid from bone ash or precipitates of calcium phosphate. However, regardless of whether calcium carbonate is adsorbed on the bone or is part of the apatite lattice, the fact remains that the  $\text{CO}_3:\text{Ca}$  ratio of the precipitate is a reflection of the  $\text{CO}_3:\text{PO}_4$  ratio of the supernatant fluid.

In view of the above considerations, it should be interesting to examine the changes in bone  $\text{CO}_3:\text{Ca}$  ratio in relation to the serum carbonate-inorganic phosphate ratio, which is referred to as the  $\text{CO}_2:\text{P}$  ratio for convenience. In the experiments presented in this paper, there was a distinct relationship between the serum  $\text{CO}_2:\text{P}$  ratio and bone  $\text{CO}_3:\text{Ca}$ . In only one case was the relationship faulty, namely, on the low calcium-high phosphorus diet without vitamin D. The slight deviation observed may be explained if we consider the  $\text{Ca}:\text{P}$  ratio of the bone. In this group (as will be shown in a subsequent publication), the residual  $\text{Ca}:\text{P}$  ratio was 1.33, while in the other six groups this ratio ranged from 1.48 to 1.52.

Let us examine the variations in bone carbonate reported in the literature in the light of differences in the serum  $\text{CO}_2:\text{P}$  ratio. An increase in the proportion of bone carbonates has been reported in rachitic compared with normal bone (4, 6). This rickets was produced on a high calcium-low phosphorus diet (so-called "low phosphate rickets"). The serum inorganic phosphorus in such cases should be markedly reduced (17-19). Thus, the increased bone carbonate may be related to the increased serum  $\text{CO}_2:\text{P}$  which results. Had these investigators examined the bones in rickets due to low calcium-high phosphorus diets, they would not have associated the increased proportions of bone carbonate *per se* with rickets.

In our experiments, it is readily seen that the high carbonate content of the bone in Group B results from the high serum  $\text{CO}_2:\text{P}$  ratio, while the low carbonate content of the bones of Groups A and C is related to the low  $\text{CO}_2:\text{P}$  ratio in the serum. Similar observations have been reported on a calcium-poor diet in swine (12) and also in rats (8). Brooke *et al.* (8) concluded that in the rats kept on a calcium-poor diet the calcium lost is mainly the calcium carbonate of the bone salts. These conditions are analogous to our low calcium diet groups (A and C). From previously mentioned considerations (17-19) and on the basis of the serum  $\text{CO}_2:\text{P}$  ratio found in Groups A and C, it seems justifiable to apply the explanation used to account for the low carbonate content in those groups to Brooke's results.

When acid rations were fed to swine (10, 12), a decreased bone carbonate content was reported. The resulting acidosis lowers the serum  $\text{CO}_2\text{:P}$  ratio and in this respect may be linked with the low bone carbonate. When mineral acid is fed to rats (9), the decreased bone carbonate found may also be associated with the lowered serum  $\text{CO}_2\text{:P}$  ratio associated with the resulting acidosis.

In prolonged starvation a condition of acidosis sets in, accompanied by a rise in serum inorganic phosphorus (30, 31). Accordingly the reduced carbonate content of the bone by inanition may be considered a reflection of the reduced  $\text{CO}_2\text{:P}$  ratio of the serum. Thus it appears that in this case also the decreased carbonate in bone may be associated with the decreased serum  $\text{CO}_2\text{:P}$  ratio.

The decrease in bone carbonates in all the above conditions may be assigned to reduced serum  $\text{CO}_2\text{:P}$  ratios rather than to decomposition in the presence of acid, since the changes in the pH of the blood were necessarily slight.

In alkalosis produced in swine by a rachitogenic alkaline ash diet, an increased bone carbonate content is reported (12). This bone carbonate content may be ascribed to the increased serum  $\text{CO}_2\text{:P}$  ratio, since in alkalosis, the  $\text{CO}_2$  content of the serum increases.

It is well known that in rapidly growing animals the serum inorganic phosphorus is higher than in older animals (32), whereas no significant changes in serum  $\text{CO}_2$  are known. Thus, the increase in the bone  $\text{CO}_3\text{:Ca}$  ratio with age may be based on the higher  $\text{CO}_2\text{:P}$  ratio of the serum.

In the one reported case of osteopetrosis in which serum calcium, inorganic phosphorus,  $\text{CO}_2$ , and complete bone analyses were performed (14, 33), the findings may be explained by our reasoning. The bones of this patient (a  $3\frac{1}{2}$  month-old male) showed hypermineralization, high density, and high carbonate content, accompanied by severe rickets which did not respond to vitamin D or irradiation therapy. The carbonate content of the bone was high, equal to that of a normal adult bone. The serum  $\text{CO}_2$  was normal, calcium was in the low normal range, but the inorganic phosphorus was low. In this pathological condition, too, the high carbonate content of the bone may be related to the raised serum  $\text{CO}_2\text{:P}$  ratio.

The bones of certain marine fishes contain much less carbonate than those of the higher vertebrates. This is related to the lower carbonate content of their body fluids, which have a roughly equivalent phosphorus level (34). Here, too, the lower carbonate content of the solid phase may be referred back to the lowered  $\text{CO}_2\text{:P}$  ratio of the liquid phase.

The above discussion is not intended to imply that the serum  $\text{CO}_2\text{:P}$  ratio is the only factor, but it attempts to call attention to the importance

of this relationship in explaining all the observed conditions and to its basis on theoretical chemical knowledge.

It is undoubtedly of importance to know the influence inside the cell which may alter the relative proportions of carbonate to phosphate due to metabolic activity, which in turn may be influenced by factors not as yet fully understood. However, even here it is possible to predict that the carbonate content of bone is a function of the ionic activity of carbonate, phosphate, and calcium in the matrix of the cells.

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#### SUMMARY

1. A high  $\text{CO}_3\text{:Ca}$  ratio in bone was found on high calcium-low phosphorus diets, whereas this ratio in bone was lower on low calcium diets containing either suboptimum or large amounts of phosphorus.

2. Vitamin D lowered the  $\text{CO}_3\text{:Ca}$  ratio of the bones of animals on the experimental diets used.

3. The changes in bone  $\text{CO}_3\text{:Ca}$  ratio with our experimental dietary conditions and in other conditions reported in the literature are related to changes in the  $\text{CO}_2\text{:P}$  ratio of the serum. The serum  $\text{CO}_2\text{:P}$  ratios in turn are influenced by the dietary  $\text{Ca:P}$  ratios and levels, modified by vitamin D and other factors.

4. The implications of these findings on the understanding of the mechanism of bone formation are discussed.

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## THE VITAMIN B<sub>6</sub> GROUP

### VI. THE COMPARATIVE STABILITY OF PYRIDOXINE, PYRIDOXAMINE, AND PYRIDOXAL

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In previous papers the effect of heating pyridoxine with amino acids (1), ammonia, oxidizing agents (2), and various other substances on its activity for various lactic acid bacteria was reported. There was frequently an increase in growth-promoting activity for these organisms, due to formation in small yields of pyridoxal or pyridoxamine by these procedures. Because of the high activity of these compounds for these organisms relative to that of pyridoxine, such an increase in activity would appear even though the greater portion of the total vitamin B<sub>6</sub> (pyridoxine, pyridoxamine, and pyridoxal) was destroyed by the treatment. In subsequent papers of this series, behavior of these compounds toward nitrous acid (3, 4), cyanides (4), and heating with amino or keto acids (4, 5) was described.

To define further the stability of the B<sub>6</sub> vitamins, a study was made of the effect of light, acids, alkali, and oxidizing agents on pyridoxine, pyridoxamine, and pyridoxal. This was patterned after the recent study of Hochberg *et al.* (6), which dealt only with pyridoxine. The results are given below.

#### EXPERIMENTAL

Assays for total vitamin B<sub>6</sub> were obtained by a slight modification of the yeast growth method of Atkin *et al.*, with *Saccharomyces carlsbergensis* 4228 (7), which responds about equally to pyridoxine, pyridoxamine, and pyridoxal (8).

In all cases solutions of these compounds were at a concentration of 30  $\gamma$  per cc. when treated.

*Destruction by Light*—Solutions of pyridoxine, pyridoxamine, and pyridoxal were prepared in 0.02 M phosphate buffer at pH 6.8, and exposed to light in tightly corked Erlenmeyer flasks. A control sample was removed from each flask immediately prior to exposure to be assayed along with the light-treated samples.

The activity of samples following various conditions of exposure is given in Table I. All three compounds are rapidly destroyed by light; of the

three, pyridoxine is least readily destroyed. Essentially the same results were obtained on similar treatment of samples at concentrations of 0.02  $\gamma$  of the compound per cc. of solution. Exposure to light of solutions containing all three compounds gave essentially the assay values which would be predicted from knowledge of the behavior of the individual compounds.

The effect of pH on inactivation by light is shown in Table II. Of the three compounds tested, pyridoxamine alone gave evidence of marked instability in 0.1 N acid solution, although all three substances are slowly de-

TABLE I  
*Destruction of Vitamin B<sub>6</sub> by Light*

Light source	Time of exposure	Per cent inactivation		
		Pyridoxine	Pyridoxamine	Pyridoxal
	<i>hrs.</i>			
None.....	0	0	0	0
Direct sunlight*	1	85	95	>99
"    "	3	97	99	>99
Diffuse daylight†	1	7.7	29	18
"    "	3	39	70	50
"    "	6	59	89	76
Artificial light‡	1	4.5	5.0	3.9
"    "	3	15	24	13
"    "	6	26	47	33

\* Exposure was begun at 9.30 a.m. and continued as indicated.

† Placed near a window in the laboratory (northern exposure).

‡ Exposed at a distance of 8 inches from a 200 watt tungsten lamp with reflector.

stroyed. In neutral and alkaline solution, all three compounds showed marked instability. The results with pyridoxine agree with those previously reported by Hochberg *et al.* (6).

To check the possibility that inactivation resulted from light-induced oxidation, solutions were prepared in freshly boiled water, then exposed to diffuse light in an atmosphere of methane. Results (Table III) show that destruction by light occurs in the absence of oxygen. Only with pyridoxal did destruction result more rapidly when oxygen was present.

*Stability on Heating with Acid, Alkali, and Oxidizing Agents*—Like pyridoxine (6), pyridoxamine and pyridoxal are stable to heating with hydrochloric and sulfuric acids (Table IV). Pyridoxine and pyridoxamine are not destroyed by heating with strong alkali; pyridoxal shows only slight instability under the same conditions. Nitric acid rapidly destroys all three compounds, presumably by its oxidative action (6). From the re-

sults with nitric acid, it appears that pyridoxal is most stable to oxidation and pyridoxamine least stable.<sup>1</sup>

Hochberg *et al.* (6) reported no destruction of pyridoxine in alkaline solution by manganese dioxide. The amine and aldehyde were found to

TABLE II  
*Effect of pH on Destruction of Vitamin B<sub>6</sub> by Light*

pH	Per cent inactivation*		
	Pyridoxine	Pyridoxamine	Pyridoxal
13.0	>99	>99	>99
9.0	99	99	99
6.8	94	96	99
3.0	39	95	93
1.0†	30	84	27

\* Exposed 3 hours to diffuse laboratory light and 2 hours to direct sunlight.

† Exposure to 4 hours of diffuse laboratory light under these conditions resulted in less than 10 per cent inactivation of pyridoxine and pyridoxal, but 44 per cent inactivation of pyridoxamine.

TABLE III  
*Effect of Oxygen on Destruction of Vitamin B<sub>6</sub> by Light*

Exposure	Per cent inactivation*		
	Pyridoxine hydrochloride	Pyridoxamine	Pyridoxal
Oxygen present.....	49	91	76
" absent.....	56	89	55

\* Unbuffered aqueous solutions of the compounds were exposed to diffuse laboratory light for 7 hours at room temperature.

exhibit similar stability. In acid solution, however, manganese dioxide rapidly destroys all three compounds at room temperature, as does po-

<sup>1</sup> At elevated temperatures, pyridoxamine is inactivated by dissolved oxygen. This was shown by the fact that solutions prepared for assay which contained from 0.0006 to 0.006  $\gamma$  of pyridoxamine per cc. showed 65 to 85 per cent loss in activity when steamed at 100° for 10 minutes. Pyridoxine and pyridoxal were undamaged by the same treatment. If the solutions of pyridoxamine were prepared in oxygen-free water (boiled and cooled), then subjected to the same treatment, no destruction of activity was evident. This effect of dissolved air is serious only when extremely dilute solutions of pyridoxamine are heated. With solutions containing as much as 1 to 10  $\gamma$  per cc., the per cent destroyed by similar treatment is so small as to escape detection, although presumably similar absolute amounts are destroyed.



tassium permanganate.<sup>2</sup> Under conditions used herein, hydrogen peroxide destroys these compounds only at elevated temperatures. Previous reports of destruction of pyridoxine by this agent at room temperature were compli-

TABLE IV  
*Effect of Heating with Acid, Alkali, and Oxidizing Agents on Vitamin B<sub>6</sub>*

Treatment			Per cent inactivation		
Reagent	Time	Temperature	Pyridoxine*	Pyridoxamine	Pyridoxal
	hrs.	°C.			
Water.....	1	100	0	0	0
".....	0.25	121	0	0	0
Hydrochloric acid, 5 N.....	1	100	0	0	0
" " 1 ".....	0.25	121	0	0	0
Sulfuric acid, 5 N.....	1	100	0	0	0
" " 4 ".....	0.5	121	0	0	0
Sodium hydroxide, 5 N.....	1	100	0	0	17
" " 1 ".....	0.25	121	0	0	0
Nitric acid, 1 N.....	1	100	10	60	0
" " 3 ".....	1	100	22	87	31
" " 5 ".....	1	100	57	>99	62
Manganese dioxide (basic solution)†.....	1	26	0	0	0
" " (acid)‡.....	1	26	>99§	>99	>99
Potassium permanganate  .....	1	26	99§	>99	>99
Hydrogen peroxide¶.....	4	26	0§	0	0
" ".....	1	121	>99§	>99	>99

\* The values given are those reported by Hochberg *et al.* (6) except where otherwise indicated.

† 600  $\gamma$  of the compound in 20 cc. of 0.33 N sodium hydroxide were shaken with 600 mg. of manganese dioxide.

‡ 600  $\gamma$  of the compound in 20 cc. of 0.1 N hydrochloric acid were shaken with 600 mg. of manganese dioxide.

§ Data of the authors.

|| 600  $\gamma$  of the compound in 20 cc. of water plus 0.06 cc. of 4 per cent potassium permanganate were allowed to stand 1 hour. The excess permanganate was then destroyed with 1 drop of 3 per cent hydrogen peroxide, at room temperature.

¶ 600  $\gamma$  of the compound in 14 cc. of water plus 6 cc. of 3 per cent hydrogen peroxide were treated at time and temperatures indicated. The solutions were then made 0.1 N with sodium hydroxide and the excess peroxide removed by treatment with manganese dioxide. This avoided the destructive effect due to manganese dioxide when used in acidic solution.

cated by use of manganese dioxide (presumably in slightly acid solution) for removal of the excess peroxide.

\* When an excess of these reagents is avoided, much pyridoxal is formed from pyridoxine and can be detected by assay with *Streptococcus faecalis* or *Lactobacillus casei* (2, 3). One of the products of further oxidation is 4-pyridoxic acid (3, 9).

## SUMMARY

Like pyridoxine, pyridoxamine and pyridoxal are rapidly inactivated by exposure to light. Inactivation proceeds most rapidly in neutral or alkaline solution. In 0.1 N acid, all three compounds are comparatively stable to light; pyridoxamine, however, is destroyed fairly rapidly by exposure to direct sunlight even under these conditions. This inactivation by light is not dependent upon the presence of oxygen.

Pyridoxine, pyridoxamine, and pyridoxal are not inactivated by heating at 100° with 5 N sulfuric or hydrochloric acids. Pyridoxal displays slight sensitivity to alkali under these conditions; pyridoxamine and pyridoxal do not. All three compounds are rapidly destroyed by oxidizing agents.

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## THE VITAMIN B<sub>6</sub> GROUP

### VII. REPLACEMENT OF VITAMIN B<sub>6</sub> FOR SOME MICROORGANISMS BY *d*(-)-ALANINE AND AN UNIDENTIFIED FACTOR FROM CASEIN

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Previous studies with *Streptococcus faecalis* R (*S. lactis* R) have shown that this organism ordinarily requires vitamin B<sub>6</sub> for growth (1, 2), but that this growth factor is not required if sufficient alanine is added to the medium (3). It was postulated that alanine served as a precursor of vitamin B<sub>6</sub> for this organism, and that organisms which were able thus to utilize alanine synthesized the remaining portion of the vitamin B<sub>6</sub> molecule, and coupled it with alanine. *Lactobacillus casei*, which also requires vitamin B<sub>6</sub> for growth, was unable to dispense with it even in the presence of excess alanine. It was later reported (4) that, although the complete growth response of *L. casei* to vitamin B<sub>6</sub> could not be elicited by alanine, some effect was evident, since growth in the blank tubes (which contained no added vitamin B<sub>6</sub>) was significantly higher when *dl*-alanine was added. This behavior would be explicable if failure of *L. casei* to utilize alanine in place of vitamin B<sub>6</sub> were due to its inability to synthesize the remaining portion of the vitamin B<sub>6</sub> molecule, and if limited amounts of this hypothetical second precursor were present in cells of the inoculum.

To test one phase of this hypothesis required only that various natural materials be assayed with *Lactobacillus casei* for their apparent content of vitamin B<sub>6</sub> in the presence and absence of added alanine. When this was done, it was found that most materials showed little or no difference. An enzymatic digest of vitamin-free casein, however, proved to be over 30 times as potent when tested in the presence of *dl*-alanine as it was in the absence of this substance. When such a digest was added to the basal medium in amounts too small to produce a growth effect alone, *dl*-alanine replaced vitamin B<sub>6</sub> for *Lactobacillus casei*, as it did for *Streptococcus faecalis*. A comparison of the *d* and *l* isomers of alanine showed that *d*(-)-alanine was far more effective for both organisms in producing this response than was its naturally occurring antipode. On the other hand, when an alanine-free medium was supplemented with vitamin B<sub>6</sub>, *l*(+)-alanine was more active than *d*(-)-alanine in promoting growth. Some details of these findings are presented below.

## EXPERIMENTAL

*Testing Procedures*—*Streptococcus faecalis* R and *Lactobacillus casei* were used for the growth tests in accordance with previously described techniques (2), modified when necessary as described below.

*Effect of dl-Alanine on Response of Lactobacillus casei to Enzymatic Digest of Casein in Vitamin B<sub>6</sub>-Free Medium*—10 gm. of vitamin-free casein (Labco) were suspended in 200 cc. of 0.8 per cent sodium bicarbonate solution. An aqueous suspension containing 50 mg. of pancreatin (Armour's) and 50

TABLE I  
*Effect of dl-Alanine on Response of Lactobacillus casei to Enzymatic Digest of Casein in Vitamin B<sub>6</sub>-Free Medium*

Addition to basal medium*	Amount per 10 cc.	Galvanometer reading†	
		N dl-alanine	1 mg. dl-alanine per 10 cc.
	mg.		
None.....		10	13
		10	12
Pyridoxine hydrochloride.....	0.0002	31	32
	0.002	63	64
	0.01	90	90
Enzymatic casein digest.....	0.3		32
	1.0	11	45
	3.0	14	60
	10	21	94
	30	40	
	50	67	

\* The basal medium and cultural conditions were those previously given for *Lactobacillus casei* (2). The basal medium contains 0.5 per cent of a casein hydrolysate as source of amino acids, and therefore contains some l(+)-alanine. The incubation time was 20 hours at 37°.

† Distilled water reads 0.0; a reading of 100 corresponds to no light transmitted.

mg. of trypsin (1:110) was added, and the mixture incubated under toluene at 37° for 8 days. It was then adjusted to pH 6.0, heated at 100° for 15 minutes, filtered, and diluted to 500 cc. To remove traces of vitamin B<sub>6</sub> which might be present, the solution was stirred for 30 minutes with 20 gm. of activated charcoal (Darco G-60) and filtered. The effect of this digest upon the growth of *Lactobacillus casei* in a vitamin B<sub>6</sub>-free medium is shown in Table I. The digest is over 30 times as potent in growth-promoting action when dl-alanine is added as it is when dl-alanine is absent. Pyridoxine shows no such differences in growth-promoting action in the absence and presence of dl-alanine. Subsequent experiments showed that

this was also true for pyridoxamine and pyridoxal. These data demonstrate the presence in enzymatic digests of casein of one or more substances which, together with alanine, effectively replace vitamin B<sub>6</sub> for growth of *L. casei*.<sup>1</sup> The active substance (or substances) is rapidly and completely

TABLE II

*Growth Response of Lactobacillus casei to dl-Alanine and Related Substances on Vitamin B<sub>6</sub>-Free Medium\**

Addition to basal medium*	Amount added per 10 cc.	Galvanometer reading†	Relative potency (approximate)
	mg.		
None.....		20	
Pyridoxine hydrochloride.....	0.10	92	
dl-Alanine.....	0.050	45	
	0.10	69	
	0.15	78	1.0
	0.50	84	
	1.0	84	
l(+)-Alanine‡.....	0.10	22	
	0.50	24	0.0075
	2.0	27	
	4.0	35	
d(-)-Alanine‡.....	0.025	45	
	0.050	68	
	0.10	79	
	0.15	84	2.0
	0.20	84	
Sodium pyruvate.....	1.0	20	0.00012
	3.0	23	
	10.0	26	

\* The basal medium was that described in Table I, supplemented with 10 mg. per 10 cc. of the charcoal-treated, enzymatic casein digest (see text).

† As in Table I.

‡ The l(+)- and d(-)-alanines were analytically pure samples prepared by resolution of dl-alanine and were kindly supplied by Professor Max S. Dunn. The preparation and physical constants of these samples have been reported in detail by Dunn and coworkers (9). The same preparations were used throughout the investigation.

destroyed by treatment with strong acids or alkali, is adsorbed by charcoal only with great difficulty, and is not destroyed by exposure to direct sun-

<sup>1</sup> At comparatively high levels, the casein digest replaces vitamin B<sub>6</sub> in the absence of dl-alanine. This may be due to the presence of traces of vitamin B<sub>6</sub> in the digest, or an effect of the unknown substance when present in excess. Separate experiments have shown that a large number of lactic acid bacteria which otherwise require added vitamin B<sub>6</sub> will grow well in its absence if an enzymatic protein digest is included in the medium; in many cases the response is improved by added dl-alanine.

light for a period of several hours. In all these respects, it differs markedly from pyridoxine, pyridoxamine, or pyridoxal (5). It can be differentiated from the substance present in enzymatic protein digests which promotes early growth of *L. casei* and other lactic acid bacteria in media which contain pyridoxine (6-8) on the basis of its distribution. Its nature is being further investigated.

TABLE III

*Growth Response of Streptococcus faecalis to dl-Alanine and Related Substances on Vitamin B<sub>6</sub>-Free Medium*

Addition to basal medium*	Amount added per 10 cc	Galvanometer reading†	Relative potency (approximate)
	mg		
None . . . . .		12	
Pyridoxine hydrochloride. . .	0.10	73	
dl-Alanine	0.10	24	
	0.30	35	
	1.0	70	1 0
	3.0	76	
l(+)-Alanine . . . . .	0.50	30	
	1.0	46	
	3.0	65	0 36
	10.0	75	
d(-)-Alanine . . . . .	0.03	18	
	0.10	29	
	0.30	58	2 0
	1.0	77	
Sodium pyruvate . . . . .	1.0	17	
	3.0	22	0.02
	10	26	

\* The basal medium and cultural conditions were those previously given for *Streptococcus faecalis* R (2). This medium contains 0.5 per cent of a casein hydrolysate as source of amino acids, and therefore contains some l(+)-alanine, which is essential for growth of the test organism (3), cf. also Table IV). Incubation time, 16 hours at 30°.

† As in Table I.

*Comparative Growth Response of Lactobacillus casei and Streptococcus faecalis to Optical Isomers of Alanine in Vitamin B<sub>6</sub>-Free Media*—Consideration of Table I reveals that, under the conditions used, *Lactobacillus casei* grows only slightly in the vitamin B<sub>6</sub>-free medium supplemented with 10 mg. of the enzymatic casein digest per 10 cc., but grows very profusely when 1 mg. of dl-alanine is also added. When the enzymatic digest is added to the medium (containing no vitamin B<sub>6</sub>) at this concentration level, alanine becomes the factor limiting growth. The response of *Lactobacillus casei*

to *dl*-alanine and its optically active constituents under these conditions is shown in Table II. The surprising fact emerges that *d*(-)-alanine is the substance which promotes growth under these conditions. *l*(+)-Alanine is less than 1 per cent as active as its antipode; pyruvic acid has still less activity. With pyruvic acid the full response obtained with *d*(-)-alanine cannot be obtained at any concentration.

Similar data obtained with *Streptococcus faecalis* R are presented in Table III. The enzymatic digest of casein is unnecessary to secure a re-

TABLE IV

*Growth Response of Streptococcus faecalis* R to Optical Antipodes of Alanine in Alanine-Free Medium Containing Vitamin B<sub>6</sub>

Addition to basal medium*	Amount added per 2.5 cc.	Galvanometer reading	Relative potency† (approximate)
	mg.		
None.....	0	4.0	
<i>dl</i> -Alanine.....	0.04	18.0	
	0.06	43.5	
	0.09	55.8	
	0.12	58.0	1.0
	0.18	59.0	
<i>l</i> (+)-Alanine.....	0.04	27.5	
	0.06	54.0	1.3
	0.09	58.0	
<i>d</i> (-)-Alanine.....	0.10	22.3	
	0.15	24.6	
	0.20	40.0	0.33
	0.30	55.0	
	0.50	59.3	

\* The basal medium and technique used were essentially those described by McMahan and Snell (11). The medium was modified as follows: additional pyridoxine (25  $\gamma$  per 2.5 cc.) and additional K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> (6.25 mg. each per 2.5 cc.) were added to the medium; alanine was omitted.

† In a series of subsequent assays *d*(-)-alanine showed potencies of 0.40, 0.36, 0.42, and 0.30; *l*(+)-alanine potencies of 1.20, 1.35, and 1.27 with respect to *dl*-alanine (potency 1.0). Pyruvic acid was inactive.

sponse to alanine from this organism, and was not added to the medium. With this organism, *d*(-)-alanine is from 6 to 10 times more active than *l*(+)-alanine in substituting for vitamin B<sub>6</sub>. In contrast to *Lactobacillus casei*, however, this organism is also able to make effective use of *l*(+)-alanine. Pyruvic acid has very slight activity, and does not give the full response given by alanine at any concentration. Addition of ammonium salts did not increase availability of pyruvic acid for either organism.

*Comparative Growth Response of Streptococcus faecalis* R to Optical Isomers



*of Alanine in Alanine-Free Medium Containing Vitamin B<sub>6</sub>*—When vitamin B<sub>6</sub> is present, alanine is not required for the growth of *Lactobacillus casei* (10). *Streptococcus faecalis* R, however, requires this amino acid for growth in the presence of vitamin B<sub>6</sub> (3). The effectiveness of the optical antipodes of alanine in permitting such growth was therefore determined. Results (Table IV) show that under these conditions *l*(+)-alanine is considerably more active in promoting growth than is *d*(-)-alanine. This result would be expected, since alanine is presumably required in this case for formation of proteins, and only *l*(+)-alanine is thought to occur naturally in proteins.

#### DISCUSSION

Results cited above are most readily interpreted as indicating that *d*(-)-alanine and one or more substances of unknown nature present in an enzymatic digest of casein serve as precursors from which vitamin B<sub>6</sub> is synthesized by these microorganisms (*cf.* (3,4)). If this is true, the synthetic process must be rather inefficient, since large amounts of *d*(-)-alanine are required compared with the amounts of pyridoxamine or pyridoxal which permit growth of these organisms (2). It is possible, of course, that presence of these substances permits growth of the organisms, and even synthesis of vitamin B<sub>6</sub> without their direct involvement as essential precursors in the synthesis, but this is considered less likely. However one interprets the effect, two points of major interest arise from the investigation: (a) the discovery of a substance of unknown nature (presumably an amino acid or peptide) in protein digests which is physiologically active for some organisms, and establishment of test conditions for detecting its presence, and (b) the demonstration that amino acids of *d* configuration may be necessary for processes essential for growth of living organisms. Previous investigations such as those dealing with *d*-amino acid oxidase (12) or the natural occurrence of *d*-amino acids (13-15), including *dl*-alanine (16), in products of microbiological origin have indicated that *d*-amino acids may occur naturally under some conditions; but so far as the author is aware, no previous data indicating that such substances may be essential for normal metabolic processes have been obtained.

The fact that only 1 mg. of *dl*-alanine would produce physiological effects of the observed magnitude when added to a basal medium which already contains more than this amount of *l*(+)-alanine is satisfactorily explained by the lack of activity of the latter substance. It is interesting that for *Streptococcus faecalis*, pyruvic acid does not replace either *d*(-)- or *l*(+)-alanine in their respective functions, although the optical antipodes are utilized with low efficiency. If only *l*(+)-alanine serves for protein synthesis, and if only *d*(-)-alanine serves for replacing vitamin B<sub>6</sub> (as is indicated by the specificity of its requirement by *Lactobacillus casei*), then

pyruvic acid must not appear as an intermediate in the interconversion of these two substances.

#### SUMMARY

An enzymatic digest of vitamin-free casein contains a substance which, together with *dl*-alanine, permits growth of *Lactobacillus casei* in the absence of vitamin B<sub>6</sub>. In contrast to vitamin B<sub>6</sub>, this substance is destroyed by acid or alkaline hydrolysis, is not destroyed by light, and is not readily adsorbed from aqueous solutions by charcoal.

When optimal levels of this substance are added to the vitamin B<sub>6</sub>-free medium, the growth response of *Lactobacillus casei* to alanine can be determined. It was found that under these circumstances *d*(-)-alanine promotes growth, while *l*(+)-alanine is almost inactive.

For *Streptococcus faecalis* R, alanine alone replaces vitamin B<sub>6</sub>. Although *l*(+)-alanine has some activity in this respect, it is less than one-sixth as active as *d*(-)-alanine. If the growth response of the same organism to the optical antipodes of alanine is determined in an alanine-free medium which contains vitamin B<sub>6</sub>, it is found that *l*(+)-alanine is most active, while *d*(-)-alanine has considerably less activity. It thus appears that both optical forms of alanine are required for growth of these organisms in the absence of vitamin B<sub>6</sub>, each serving different functions within the organism.

A short discussion of these results is included.

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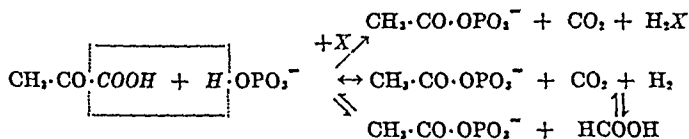
# ON THE CONDENSATION OF ACETYL PHOSPHATE WITH FORMATE OR CARBON DIOXIDE IN BACTERIAL EXTRACTS\*

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A conversion of the acetyl part of pyruvate to acetyl phosphate first became apparent in pyruvate dehydrogenation with an enzyme from a *Lactobacillus delbrueckii* (1, 2). Recently a more general application of this scheme of pyruvate degradation was observed by Koepsell, Johnson, and Meek (3) and by Utter and Werkman (4). The newly described reactions are straight cleavages of a pyruvate-phosphate addition product to acetyl phosphate plus either hydrogen and carbon dioxide or formate. The relationship between the three types of pyruvate degradation known to yield acetyl phosphate appears in the following equations.



In the above reactions cleavage is coupled with condensation and, from thermodynamic considerations (*cf.* (5)), it appeared likely that in spite of a fission between the 2 carbon atoms this type of reaction represented a reversible shift of energy rather than an energy loss to the environment.

For an experimental approach to the problem of reversibility the two more recently discovered reactions, occurring in *Clostridium butylicum* (3) and *Escherichia coli* (4) respectively, were especially promising.<sup>1</sup> There we encounter the simplest type of phosphoroclastic reaction, the one which is essentially the result of intramolecular rearrangement and does not require an external impulse. The dehydrogenation of pyruvate in *Lactobacillus delbrueckii*, on the other hand, is constructed in such a manner that

\* This work was supported by a grant from the Commonwealth Fund.

<sup>1</sup> Although *Clostridium butylicum* and *Escherichia coli* both catalyze the reaction pyruvate  $\rightleftharpoons$  acetyl phosphate, hydrogen, and carbon dioxide, the enzymatic mechanism in the two organisms is not identical. Koepsell and Johnson reported that *Clostridium* extracts, which catalyze the over-all reaction, do not react with formate. In contrast, with appropriately grown *Escherichia coli*, the reaction occurs in two distinct stages, (1) pyruvate  $\rightleftharpoons$  acetyl phosphate, formate; (2) formate  $\rightleftharpoons$  hydrogen, carbon dioxide.

only in the presence of a hydrogen acceptor is the phosphoroclastic split of pyruvate allowed to proceed (6). There a reversal can be expected only in the presence of a suitable hydrogen donator, which as an additional component brings the system into a higher order of reaction.

Of these phosphoroclastic pyruvate degradations, moreover, the split to acetyl phosphate and formate in *Escherichia coli* (4) is the simpler one. Assuming the still somewhat problematical pyruvate-phosphate addition product to be a single component, this reaction may be considered in fact as a monomolecular reaction. It is with this reaction also that the most complete evidence of reversibility has been obtained so far.

In the present communication are reported experiments with preparations of *Clostridium butylicum* and *Escherichia coli* which pertain to the reversibility of the phosphoroclastic reaction. A preliminary report on part of this work appeared earlier in this *Journal* (5).

### *Methods and Enzyme Material*

*Manometric measurements* were carried out in the usual manner with manometers and vessels of the Warburg type.

*Phosphate* was determined according to Fiske and Subbarow (7), and *acetyl phosphate* as described in an earlier publication (8). In the experiments with radioactive phosphate we used, for a separation of inorganic and acetyl phosphate fractions, the method previously described (8) under the heading, "Direct determination of acetyl-bound phosphate." The calcium phosphate fractions thus obtained were dissolved in hydrochloric acid, made up to volume, and aliquots were used for phosphate and radioactivity assays.

*Radioactivity Determination*—Radioactive phosphorus was kindly supplied to us by Professor Irvine of the Massachusetts Institute of Technology in the form of inorganic phosphate. The samples were measured into porcelain disks and dried slowly and evenly on electric hot-plates. Under standardized conditions the rate of discharge of a quartz fiber electroscope (model 1, Lauritsen electroscope, made by the Fred C. Henson Company, Pasadena, California) was measured as described by Cohn and Greenberg (9).<sup>2</sup>

*Keto acid* was determined by the micromethod of Lu (10) as modified recently by Friedemann and Haugen (11). The phenylhydrazone was extracted with benzene, the use of which, according to Friedemann and Haugen, makes the procedure rather specific for pyruvate.

*Enzyme Preparations*—Extracts of *Escherichia coli* were prepared by the glass powder procedure of Wiggert *et al.* (12). A sample of the organism used by Utter and Werkman in their work on pyruvate metabolism (4)

<sup>2</sup> We are indebted to Dr. Waldo Cohn and Dr. Austin Brues for most valuable help with the estimation of radioactivity.

was kindly supplied to us by Professor C. H. Werkman. The culture fluid was composed as follows: 0.4 per cent beef extract, 0.4 per cent peptone, 0.2 per cent yeast extract, and 0.2 per cent NaCl in 10 per cent tap water. The pH came to about 8. A few crystals of *n*-octadecyl alcohol were added to prevent foaming. In order to prevent formation of the undesirable formate-splitting enzyme, the medium was vigorously aerated during incubation (Stephenson (13), Woods (14)). After a growth period of 24 hours at 28–30° we centrifuged the bacteria in a cream separator. Frequently the paste was frozen overnight before the extract was made. The extracts were kept frozen for several days, generally without change of

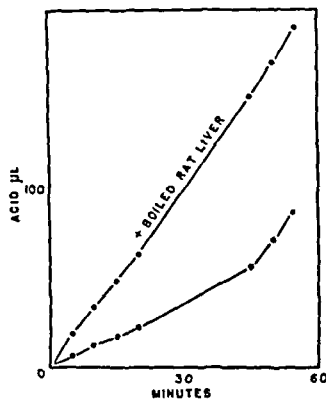


FIG. 1

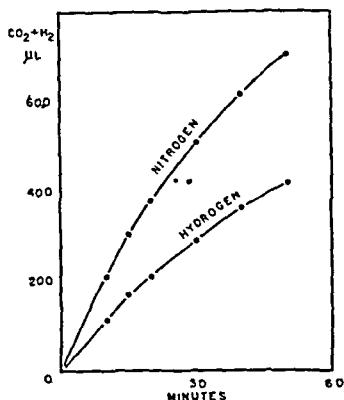


FIG. 2

FIG. 1. Induction period with pyruvate breakdown in *Escherichia coli* extract. 0.5 ml. of extract in 0.63 ml. of 0.04 *N* sodium bicarbonate and 0.016 *M* phosphate solution. At zero time, 0.05 ml. of molar pyruvate solution was dipped from the annex of the Warburg vessel. Nitrogen-10 per cent carbon dioxide in the gas phase; temperature 30.5°. Upper curve, 0.2 ml. of boiled rat liver (1:1) added.

FIG. 2. Inhibition of pyruvate decomposition by hydrogen in extract of *Clostridium butylicum*. The main compartment of the vessel contained 25 mg. of enzyme, 200 micromoles of pyruvate, and 60 micromoles of  $\text{KH}_2\text{PO}_4$  in 1.1 ml. of fluid. Nitrogen or hydrogen in the gas phase, temperature 37°. The average inhibition through hydrogen amounts to 42 per cent.

activity. Activity was tested manometrically by measuring in bicarbonate solution at 37° the acid formation due to pyruvate decomposition. 1 ml. of extract produced from 300 to 1000 microliters of acid per hour (corrected for carbon dioxide retention).

Some of the extracts, in particular those repeatedly frozen, reached the final rate of pyruvate decomposition only after prolonged incubation. This induction phenomenon is shown in Fig. 1. It indicates that an essential part of the enzyme system is activated autocatalytically. Addition of

boiled rat liver, but not of cocarboxylase alone, abolished the induction period without affecting the final rate (cf. Fig. 1). The liver extract contained various metabolites, especially appreciable amounts of carbohydrate. Therefore, its addition in reversibility experiments, although desirable, appeared impracticable.

The enzyme preparation from *Clostridium butylicum* was a sample of lyophilized extract (15) prepared by Dr. Koepsell and Dr. Johnson and generously supplied to us.

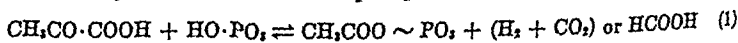
### *Inhibition of Phosphoroclastic Split of Pyruvate by Hydrogen*

In the course of a study on the action of carbon monoxide in butyric acid fermentation, Kubowitz observed (16) that hydrogen inhibited the gas formation from glucose by *Clostridium butylicum*. It seemed desirable now to test the effect of hydrogen on the isolated phosphoroclastic system of Koepsell and Johnson (15).

The experiment of Fig. 2 shows that hydrogen of atmospheric pressure causes a 40 per cent inhibition of carbon dioxide-hydrogen formation from pyruvate. In a similar experiment acetyl phosphate was determined. In 105 minutes, 12.6 and 8.5 micromoles had been accumulated in nitrogen and hydrogen respectively. The results show that the rate of enzymatic decomposition of pyruvate to acetyl phosphate, hydrogen, and carbon dioxide is slowed down considerably by hydrogen, one of the products of the reaction.

### *Exchange of Inorganic and Acetyl-Bound Phosphate*

Reversibility of a reaction of the phosphoroclastic type<sup>3</sup>



involves a continuous shift between inorganic and acetyl phosphate. Since methods for the separation of acetyl and inorganic phosphate (8) are available, such phosphate exchange can be explored with the help of radioactive phosphate.

The general procedure of these experiments was to bring the enzyme with all additions, except acetyl phosphate, into the main part of a Warburg vessel and into the annex a mixture of acetyl and radioactive inorganic phosphate. The vessel was then filled with the desired gas mixture, brought to temperature equilibrium, and the experiment was started by dipping the contents of the annex into the main part of the vessel. The experiment was terminated by addition of trichloroacetic acid to the cooled vessel.

<sup>3</sup> The sign  $\sim$  is used to distinguish the energy-rich phosphate bond with an average bond energy of 12 kilocalories from the ordinary ester bond with around 3 kilocalories (17).

In preliminary experiments, it was found that under our conditions the sum of inorganic and acetyl phosphate remained constant, inside the limits of experimental error. Moreover, all radioactivity added as inorganic phosphate was at the end recovered in the two fractions of inorganic and acetyl phosphate. Under such conditions, the degree of turnover may be estimated as exchange percentage from chemical ( $P_{ac}^{chem}$ ) and radioactivity ( $P^*$ ) determinations on the acetyl phosphate ( $P_{ac}^{chem}$ ,  $P_{ac}^*$ ) and inorganic

TABLE I

*Exchange of Radioactive P in Extracts of Clostridium butylicum*

Experiment 1, 0.3 ml. of 10 per cent enzyme solution in 0.8 ml. of water, 37°; Experiment 2, 0.4 ml. of 10 per cent enzyme solution in 0.9 ml. of water, 37°; plus additions as indicated in the table.

Experiment No	Incubation time	Gas phase (+18 per cent CO <sub>2</sub> )	$P_{ac}^*$	$P_i^*$	$P_{ac}^*$	$P_{ac}^{chem}$	Exchange
	min		microcuries	microcuries	per cent	per cent	per cent
1	3	H <sub>2</sub>	31.5	114.5	21.5	64†	34
		N <sub>2</sub>	30.5	113	21	62	34
	10	H <sub>2</sub>	36	121	23	57	40
		N <sub>2</sub>	33	115	22	59	37
2	0		9	510	2	37	(5)‡
	28	H <sub>2</sub>	133	410	25	24§	104
			130	400	24	31	77
		N <sub>2</sub>	135	410	25	32	78

†  $P_{ac}^{chem} + P_i^{chem}$  is 1.49 mg. of P.

‡ This is not real exchange, but due to experimental error in our method of fractionation.

§  $P_{ac}^{chem} + P_i^{chem}$  is 1.10 mg. of P.

( $P_i^{chem}$ ,  $P_i^*$ ) phosphate fractions respectively. Then the degree of exchange is expressed by the following relation.

$$\text{Exchange \%} = \frac{\frac{P_{ac}^*}{P_{ac}^* + P_i^*}}{\frac{P_{ac}^{chem}}{P_{ac}^{chem} + P_i^{chem}}} \times 100 \quad (2)$$

In Tables I, II, and III the results of these experiments are brought together. It appears that with both bacterial extracts, which respectively catalyze the two reactions summarized in equation (1), exchange occurs between acetyl and inorganic phosphate. Equilibrium between organic and inorganic phosphate was reached after  $\frac{1}{2}$  to 1 hour of incubation.

It is remarkable that the rate of exchange in both cases is influenced none



or little by the addition of reactants other than acetyl phosphate; e.g., hydrogen, carbon dioxide, or formate (cf. equation (1)). With *Clostridium* extract almost no acceleration was found with hydrogen in the gas phase (Table I). With extracts of *Escherichia coli*, correspondingly, formate had no or little effect on the rate of phosphate exchange (Table III). Deter-

TABLE II

*Exchange with Different Concentrations of Clostridium butylicum Extract*

5 per cent dry preparation	Exchange*	Remarks
ml.	per cent	
0.05	3.5	10 min. incubation, 37°; nitrogen in gas phase
0.15	12.5	
0.4	42.5	

\* Corrected for the blank. For further detail see Table I.

TABLE III

*Phosphate Exchange with Extracts of Escherichia coli*

0.5 ml. of bacterial extract was used per experiment in 1.25 ml. of 0.04 M fluoride, 0.04 M sodium bicarbonate, and additions as indicated in the table. Temperature 30.5°. Nitrogen-5 per cent carbon dioxide in the gas phase. Formate, when added, 0.04 M final concentration.

Experiment No.	Time	Formate	P <sub>ac</sub> <sup>*</sup> (corrected for the blank)	p <sub>chem.</sub> ac	P <sub>ac</sub> <sup>*</sup>	Exchange
	min.		millimicrocuries	per cent	per cent	per cent
1	5	—	11	62.5	2.7	4.3
		+	17	61	4.1	6.7
	20	—	55	49	13.4	27.3
		+	66	49	16	32.6
2	20	—	61	48.5	15.6	33
		+	57	53.6	15	27
	40	—	78	38.4	20.5	53.3
		+	94	42	24.7	59

p<sub>chem.</sub> ac + P<sub>i</sub> 1.28 and 1.38 mg. of P for Experiments 1 and 2 respectively;  
P<sub>ac</sub><sup>\*</sup> + P<sub>i</sub><sup>\*</sup> 410 and 380 millimicrocuries for Experiments 1 and 2.

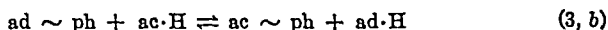
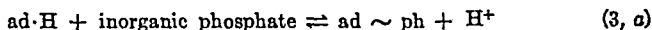
mination of formate by the mercurous chloride method indicated, however, the presence of 5.4 micromoles of formate per ml. of *Escherichia coli* extract.

The extracts used in these experiments contained, of course, a great variety of enzyme systems besides the one under investigation. Besides the pathway indicated by the reversible phosphoroclastic reaction, therefore, the possibility of other routes of exchange has to be considered. It

has been shown previously with *Clostridium* extracts that a reversible phosphoryl transfer occurs between acetyl (ac ~ ph) and adenyl polyphosphate (ad ~ (ph)<sub>z</sub>). On the other hand Meyerhof *et al.* (18) demonstrated in muscle extract a rapid exchange between inorganic and adenyl polyphosphate due to the coupling with lactic acid fermentation. Therefore a coupling of the reactions indicated in equations (3, a) and (3, b) comes into consideration as an alternative course of exchange.

fermentation

↓



An important difference between exchange through condensation (equation (1)) and through a coupling sequence is that the latter involves the phosphorylation of free acetic acid (equation (3, b)). If this were the main cause of the exchange observed here between inorganic and acetyl phosphate, an equally fast exchange between free and phosphorylated acetic acid should be expected. Utter, Lipmann, and Werkman (19) have with equivalent extracts of *Escherichia coli* determined the exchange between acetic acid containing heavy carbon and pyruvic acid which is expected to occur through the sequence of reactions (3, b) and (1). They find a significant entrance of acetic acid into pyruvic acid only with addition of excess adenyl pyrophosphate. Even then they found this exchange to be about 20 times slower than exchange between formic and pyruvic acids. Complete equilibrium between heavy carbon formate and pyruvate was generally reached in about 50 minutes. The highest value for the incorporation of acetate into pyruvate, however, was 0.22 out of 3.6 atom per cent excess C<sup>13</sup> in 75 minutes.

It appears then that the fast formate-pyruvate equilibration, certainly due to reaction (1), is of a velocity comparable to that of the phosphate-acetyl phosphate exchange and that the acetate exchange, expected to be due to a combination of reaction (3, b) with reaction (1), is a much slower process and dependent on the presence of an excess of adenyl pyrophosphate.

This makes a coupling of the type represented in equations (3, a) and (3, b) unlikely as a cause of our fast turnover. The combined results lead us to the conclusion that the phosphate exchange is caused predominantly by reaction (1), in which free acetic acid does not enter into the equilibrium. A reversible condensation of acetyl phosphate with compounds other than formate, or hydrogen plus carbon dioxide, however, may merit consideration as a contributing factor.

*Condensation of Acetyl Phosphate and Formate to Pyruvate*

During the experiments with *Clostridium* extracts it had been observed that just detectable amounts of a bisulfite-binding substance were formed on addition of acetyl phosphate. At that time the carbonyl formation was not followed more closely. The publication of the experiments of Kalnitsky and Werkman (20) and of Utter and Werkman (4) on the mechanism of pyruvate decomposition in extracts of *Escherichia coli*, however, as pointed out in the introduction, gave promise that in this system there could be found the most favorable conditions for a carboxylation of acetyl phosphate to pyruvate.

The experimental conditions were analogous to those in the experiments on phosphate exchange. Warburg vessels were used and in the main part were put the extract and stable additions, while the acetyl phosphate came into the annex. The gas phase contained nitrogen with 5 per cent carbon dioxide, and after equilibration to 37° the experiment was started by dipping. In all experiments bicarbonate was added in amounts slightly exceeding that of acetyl phosphate. This was necessary to buffer the acid formed by acetyl phosphate decomposition. Although fluoride was added to counteract this, mostly enzymatic, breakdown, a considerable decrease in acetyl phosphate concentration always took place in the course of such experiments (cf. Table IV). It was, therefore, advantageous to follow the decomposition manometrically, as the bicarbonate decomposition allowed an approximate estimation of the amounts of acetyl phosphate left at various stages of the experiment. The values given in Table IV, however, for acetyl phosphate were determined by the more accurate chemical method.

The unavoidable change of acetyl phosphate concentration made it impossible to obtain a stable equilibrium in these extracts. Corresponding to the continual decrease in acetyl phosphate concentration, the keto acid concentration, built up initially to a maximum, fell later, parallel with the more or less complete disappearance of acetyl phosphate (Table I, Experiment 1). A further proof that we were dealing here with a true equilibrium was given by experiments in which excess pyruvate was added at the beginning. Here, in the presence of acetyl phosphate and formate, pyruvate concentration fell to the equilibrium level indicated in Experiment 1 and remained constant for a time, while without acetyl phosphate pyruvate disappeared rapidly and practically completely during the same time interval. The approach to the equilibrium concentration of pyruvate from both sides is graphically reproduced in Fig. 3.

In some of the extract samples, pyruvate appeared more sluggishly and did not reach the levels obtained in the experiments recorded in Table IV. Generally, in such extracts, when tried with pyruvate, the induction phe-

nomenon mentioned earlier was observed. Such variations in activity, therefore, may be explained through initial incompleteness of the enzyme system which is indicated by induction.

In the last column of Table IV values for the equilibrium constant (toward condensation) are calculated; they average for  $k$  roughly 0.01. From this constant  $\Delta F_0$  is calculated (21) to be about +2.8 kilocalories. The values are, of course, only rough approximations. Our conditions were

TABLE IV

*Reversibility of Phosphoroclastic Reaction with Escherichia coli Extract*

0.5 ml. of enzyme extract in 1 ml. of 0.05 M sodium fluoride and 0.05 M sodium bicarbonate plus additions as indicated in the table. Temperature 30.5°. Nitrogen-5 per cent carbon dioxide in the gas phase.

The results are expressed in micromoles per ml.

Experiment No.	Time	Formate	Acetyl phosphate	Phosphate	Pyruvate	Phosphate $\times$ pyruvate Formate $\times$ acetyl phosphate $k$
	min.					
1	0	150	50	10	0.03	
	25	150	38	20	0.17	0.006
	60	150	24	34	0.14	0.013
	150	150	2	56	0.07	0.10
	60		24	34	0.03	
	150	150		10	0.04	
2	0	150	50	10	0.31*	
	10	150	43	17	0.22	0.006
	20	150	38	20	0.20	0.007
	40	150	31	27	0.20	0.011
3	0	150	50	10	0.31*	
	30	150	33	25	0.18	0.009
	30	150		10	0.10	
	30		33	25	0.10	
	30				0.05	

\* 0.27 micromole of pyruvate added at zero time.

rather unfavorable for the fixation of a final state of equilibrium. The true constant may be expected somewhat higher and  $\Delta F_0$  correspondingly lower than the given values.

Attempts were made to drive the reaction toward the pyruvate side, either through carbonyl fixation or through pyruvate reduction. Both methods proved inapplicable in the present case. Cyanide, the most powerful fixing reagent (22), inhibits the enzyme reaction according to Kalnitsky and Werkman (23). Hydroxylamine, semicarbazide, and similar compounds were found to react rapidly with acetyl phosphate, replacing

the acyl-bound phosphate. Reduction of pyruvate was tried through addition of hexose diphosphate or hydrogen gas, but with our extracts only slight reduction to lactate was observed even with high concentration of pyruvate.

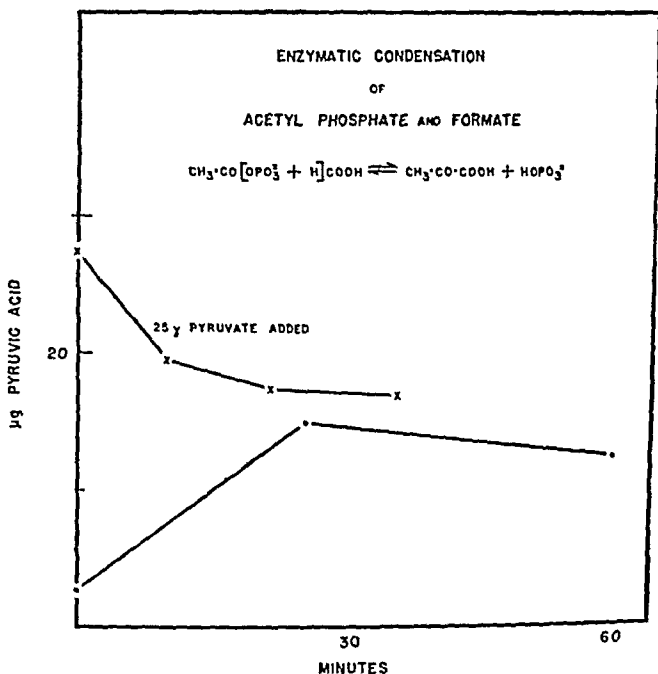


FIG. 3. The graphic reproduction of Experiments 1 and 2 of Table IV illustrates the approach to equilibrium from both sides of the equilibrium level.

### Comments

*Animal Tissues*—Carbon dioxide fixation in the carboxyl group of lactic acid has been described by Wood *et al.* (24) in pigeon liver. In their discussion of this reaction the question was left open whether it was due to  $\text{C}_3 + \text{C}_1$  or  $\text{C}_2 + \text{C}_1$  condensation or to both. Reductive carboxylation through metabolite hydrogen would offer a mechanism for  $\text{C}_2 + \text{C}_1$  condensation. The viability of such a reaction in animal tissues, however, remains to be proved. If true, this reaction would, of course, have to be considered as a pathway for the carbon dioxide fixation in glycogen as described by Vennesland *et al.* (25). Theoretically, furthermore, a rather remote possibility exists that carbon dioxide fixation in  $\alpha$ -ketoglutarate (26) may occur by reductive carboxylation of succinyl phosphate.

*Photosynthesis*—After identification of acetyl phosphate as a degradation product of pyruvic acid the possibility was mentioned (17) that a hydrogenative carboxylation may be a key reaction in photosynthesis. Our early suggestion was more recently elaborated by Ruben (27) in a discussion of the probable relation between photosynthesis and phosphorylation (cf. also (28)). The finding of Ruben and his group (29) that the primary reaction in photochemosynthesis is the formation of a carboxylic acid lent renewed emphasis to a carboxylation scheme, an obstacle for the development of which was then, however, still the apparent irreversibility of  $\alpha$ -keto acid decarboxylation. With the present demonstration of hydrogenative carboxylation of acetyl phosphate the biological possibility of such a reaction is shown, and the way is opened for a serious discussion of the part reactions of this type are likely to take in photochemosynthesis.

The recent development in photosynthesis (van Niel (30), Gaffron (31)) showed with increasing clarity that the light-promoted synthesis of carbohydrate from carbon dioxide and water may be separated into two largely independent sequences of reactions: the light reaction and the chemosynthesis. The light reaction proper emerged as a photolysis of water which feeds hydrogen into the chemosynthesis system. If, therefore, an abundant availability of photolytic hydrogen is taken for granted, the light process can be left out of consideration in this first approximation to a mechanism of chemosynthesis.<sup>4</sup>

As shown in the experimental part, at equilibrium a relatively small part of acetyl phosphate only is condensed with hydrogen and carbon dioxide, or with the energetically equivalent formate, to form pyruvic acid. However, a coupling of this reaction with the highly exergonic hydrogenation of the keto acid to hydroxy acid ( $\Delta F_0$ ;  $-11.4$  kilocalories (32)) should drive the condensation easily to completion. As an example for the occurrence of this coupling the earlier findings of Slade *et al.* (33) may be cited here. They found in *Clostridia* and *Streptococcus paracitrovorus* a fixation of carbon dioxide in the carboxyl group of lactic acid which could not be explained by the Wood and Werkman reaction. From recent findings this carbon dioxide fixation is now explained as due to condensation of acetyl phosphate, carbon dioxide, and hydrogen, coupled with reduction of pyruvic to lactic acid.

This sequence of reactions from carboxylic to the next higher hydroxy •

<sup>4</sup> Photolytic production and the utilization of hydrogen for chemosynthesis, apparently, are intimately connected with each other. In Gaffron's experiments with hydrogen-adapted organisms (31) in the dark, carbon dioxide reduction, although appreciable, was only a relatively small fraction of the reduction with optimum illumination. It should be mentioned here that in our abbreviated formulation we neglected the fact that the primary split of water is expected to occur between hydrogen and hydroxyl (cf. (31)).

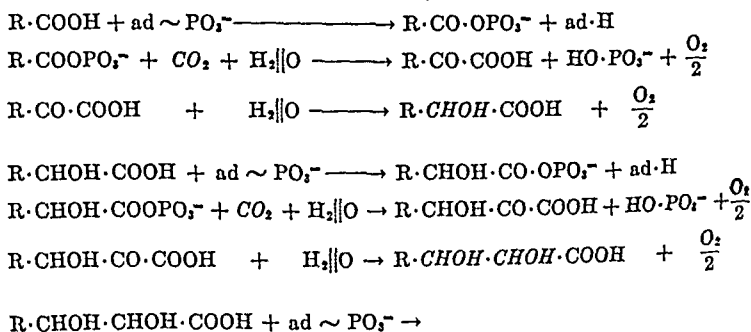
acid is summarized in Table V. The energy data show that the over-all reaction is exergonic by a large margin. Essentially this sequence represents reduction of carbon dioxide to the carbohydrate level.

TABLE V  
Thermodynamic Data for Partial Reactions of Reductive Carboxylation

Reaction	$\Delta F_0$ kilo-calories	Organism	Reference
$\text{CH}_3\text{COO}^- + \text{ad} \sim \text{PO}_3^{2-} \rightleftharpoons \text{CH}_3\text{COO} \sim \text{PO}_3^{2-} + \text{ad}^-$	+3.0	<i>Clostridium</i>	Lipmann (2)
$\text{H}_2 + \text{HCO}_3^- \rightleftharpoons \text{HCOO}^- + \text{H}_2\text{O}$	-0.2	<i>Escherichia coli</i>	Woods (14)
$\text{CH}_3\text{COO} \sim \text{PO}_3^{2-} + \text{HCOO}^- \rightleftharpoons \text{CH}_3\text{CO} \cdot \text{COO}^- + \text{HO} \cdot \text{PO}_3^{2-}$	+2.8	<i>Escherichia coli</i>	This paper
$\text{CH}_3\text{CO} \cdot \text{COO}^- + \text{H}_2 \rightleftharpoons \text{CH}_3\text{CHOH} \cdot \text{COO}^-$	-11.4	<i>Gonococcus</i>	Barron and Hastings (32)
Over-all: $\text{CH}_3\text{COO}^- + 2 \text{H}_2 + \text{CO}_2 + \text{ad} \sim \text{PO}_3^{2-} \rightleftharpoons \text{CH}_3\text{CHOH} \cdot \text{COO}^- + \text{adH} + \text{HO} \cdot \text{PO}_3^{2-}$	-5.8		

TABLE VI

Scheme of Photosynthesis by Alternating Phosphorylation and Photoreduction

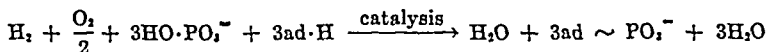


In generalization of such a sequence, a continuity of alternating reactions is formulated in Table VI, whereby long chains of  $(\text{CHOH})_x$  may be built up.<sup>5</sup> Phosphorylation of the carboxyl group, reductive carboxylation, and

<sup>5</sup> This sequence is essentially a reversal of the carbohydrate degradation by way of phosphogluconic acid through alternating dehydrogenation and decarboxylation described by Lipmann (34), Warburg and Christian (35), and Dickens (36).

further hydrogenation to hydroxy acid are aggregated into a reaction group which may be repeated indefinitely. The functioning of the scheme depends on a continuous supply of phosphate bond energy. The fraction of over-all energy which is diverted into phosphate bonds is small but of utmost importance as initiation energy. It must be provided through a side reaction by use of photolytically formed hydrogen.

Significant in this connection seems the observation of Gaffron (37) that in *Scenedesmus* algae the oxyhydrogen reaction may couple with carbon dioxide fixation. In terms of the phosphorylation-reduction scheme this coupling suggests the oxyhydrogen reaction as a source of energy-rich phosphate bonds. One energy-rich bond being the equivalent of an average of 12 kilocalories, the oxyhydrogen reaction with its 57 kilocalories can theoretically furnish maximally four to five such bonds. In practice, the oxidation of a pair of metabolite hydrogens to water was found to yield from a similar total of calories an average of three phosphate bonds (38). It has been pointed out elsewhere (39) that such multiple bond generation is necessarily a catalytic process which is largely independent of the metabolic source of the hydrogen. The process of electron transfer, from hydrogen to oxygen, is the metabolic "wheel" which generates the bonds catalytically:



In this manner for every 3 moles of carbon dioxide reduced to the carbohydrate level by 6 moles of hydrogen, 1 extra mole of hydrogen would be required to supply three energy-rich phosphate bonds. For this purpose, 1 out of 7 moles of photolytic hydrogen may be reoxidized to water.

Finally in the complete process, of course,  $(\text{CHOH})_6$  units have to be cut off continuously from the  $(\text{CHOH})_x\text{COOH}$  chain. This part could be accomplished by a reaction of the zymohexase type.

It was attempted here, and appears possible now, to devise an energetically satisfactory blue-print for the photosynthesis process by a combination of known enzymatic reactions.

#### SUMMARY

1. The decomposition of pyruvate to acetyl phosphate, hydrogen, and carbon dioxide with extracts of *Clostridium butylicum* is inhibited by hydrogen gas.

2. By use of radioactive phosphate, the exchange between inorganic and acetyl-bound phosphate is studied with extracts of *Clostridium butylicum* and of *Escherichia coli*. Rapid turnover between the two phosphate fractions was found with both extracts. This exchange is considered due to the alternating liberation and fixation of phosphate in a reversible condensation, acetyl phosphate  $\rightleftharpoons$  keto acid, phosphate.



3. Formation of  $\alpha$ -keto acid in just determinable amounts was found with extracts of *Escherichia coli* on combined addition of acetyl phosphate and formate. From our data an equilibrium constant  $k$  of approximately 0.01 obtains for the condensation, acetyl phosphate-formate  $\rightleftharpoons$  pyruvate-phosphate.

4. The significance of these reactions as partial reactions in processes of biological carbon dioxide fixation is discussed.

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# REVERSIBILITY OF THE PHOSPHOROCLASTIC SPLIT OF PYRUVATE\*

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This communication is a further study of the reversibility of the phosphoroclastic reaction in *Escherichia coli*. Previous reports (8, 17) have already indicated the reversibility of this reaction.

The fixation of carbon dioxide by its combination with a  $C_3$  compound, presumably pyruvic acid or a closely related substance, has been well established by Werkman and Wood (18). However, previous attempts to demonstrate the existence of a  $C_2 + C_1$  reaction have failed. Krampitz *et al.* (3) reported that the oxidative decarboxylation of pyruvic acid by a preparation of *Micrococcus lysodeikticus* was apparently irreversible. Evans (1) obtained similar negative results with yeast carboxylase.

Recent experiments (8, 17), however, have disclosed that a well known bacterial reaction, the hydroclastic, in fact being a phosphoroclastic split of pyruvic acid, is reversible (Reaction 1). The reversal of this reaction constitutes a  $C_2 + C_1$  addition. Although the  $C_1$  compound is formic acid rather than carbon dioxide, the formic acid is in equilibrium with carbon dioxide and hydrogen in cells of *Escherichia coli*, and thus a carbon dioxide fixation is involved.



This reaction has previously been shown to take place by the addition of phosphate (16). Lipmann and Tuttle (8) show that the energy relationships of these compounds are such that the reaction should be readily reversible.

## EXPERIMENTAL

*Escherichia coli* (E26) was grown in large quantities in a medium containing 1 per cent glucose, 0.4 per cent yeast extract, 0.8 per cent dipotassium phosphate, and 10 per cent tap water. The cells were harvested after 16 to 22 hours incubation by a Sharples centrifuge. In most cases the wet mass of cells was mixed with 2 parts of ground glass and ground by a

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† Supported by a grant from the Commonwealth Fund.

method used in this laboratory.<sup>1</sup> The bacterial extracts were frozen and used in all cases within 3 or 4 days.

The experiments were conducted in manometric flasks of 125 ml. capacity with a total volume of reaction mixture of 15 to 30 ml. at 30.4°.

C<sup>13</sup>O<sub>2</sub> was added as NaHC<sup>13</sup>O<sub>3</sub> to the main cup or side arm of the manometer flask. After the flask was filled with nitrogen or hydrogen and equilibrated, a mixture of carbon dioxide and nitrogen was obtained by tipping in a slightly acid phosphate buffer.

The HC<sup>13</sup>OOH used in these experiments was obtained by incubating a suspension of *Escherichia coli* with normal formic acid in an atmosphere of hydrogen and C<sup>13</sup>O<sub>2</sub>. After 90 to 150 minutes, depending on the concentrations of substrate used, the C<sup>13</sup>O<sub>2</sub> and HC<sup>13</sup>OOH were in equilibrium. The cells were removed by centrifugation; the mixture was acidified, aerated to remove carbon dioxide, and the HC<sup>13</sup>OOH recovered by steam distillation. The distillate was then neutralized and reduced to a small volume.

The CH<sub>3</sub>C<sup>13</sup>OOH used in some of the experiments was taken from a sample synthesized by Wood, Brown, and Werkman (19) from C<sup>13</sup>O<sub>2</sub> by means of the Grignard reaction.

Sodium pyruvate was prepared by neutralizing a solution of vacuum-distilled, commercial pyruvic acid.

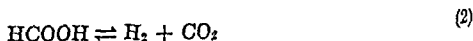
Pyruvic acid was determined by the salicylaldehyde method of Straub (15) or the ceric sulfate manometric method of Krebs and Johnson (5).

Methods of separation of the various compounds and their conversion to carbon dioxide will be described in connection with the individual experiments.

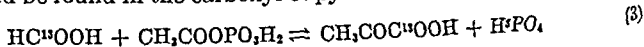
Adenyl pyrophosphate was prepared according to Needham (10).

#### *Fixation of HC<sup>13</sup>OOH in Carboxyl Group of Pyruvic Acid*

Although suspensions of *Escherichia coli* contain hydrogenlyase which catalyzes the reversible reaction between carbon dioxide and formic acids (20) (Reaction 2),



cell-free extracts prepared in the foregoing manner do not carry out this reaction. Consequently it was necessary to use HC<sup>13</sup>OOH in the demonstration of the reversibility of this reaction. If a reversal occurs, an excess of C<sup>13</sup> should be found in the carboxyl of pyruvic acid.



The experiments of Table I show this excess of C<sup>13</sup>.

Lipmann and Tuttle (8, 9) obtained pyruvic acid by incubating *Esch-*

<sup>1</sup> Kalnitsky, G., Utter, M. F., and Werkman, C. H., unpublished.

*erichia coli* extract with formic acid and acetyl phosphate. Since the equilibrium is far toward the side of formic acid and acetyl phosphate, only minute amounts of pyruvic acid were obtained. In the present experiments, fairly large amounts of pyruvate were incubated in the presence of  $\text{HC}^{13}\text{OOH}$  until one-third to two-thirds of the pyruvate had been dissimilated. If the reaction is reversible, the residual pyruvate should contain excess  $\text{C}^{13}$ . In this manner the difficulties and errors of working with very small amounts of pyruvic acid were avoided.

Previously it has been shown (16) that the principal reaction occurring in *Escherichia coli* extract on pyruvic acid is a conversion to acetic and formic acids. In addition, a small amount of lactic acid is formed by the

TABLE I  
*Fixation of  $\text{HC}^{13}\text{OOH}$  by *Escherichia coli* in  $\text{CH}_3\text{COCOOH}$*

Experiment No.	Description	Pyruvic acid utilized	Excess $\text{C}^{13}$ , atom per cent		
			$\text{NaHCO}_3$	$\text{HCOOH}$	$-\text{COOH}$ of pyruvic acid
1	<i>E. coli</i> extract added, time 60 min.	0.283	0.06	0.77 (0.94 mm)	0.78 (0.58 mm)
2	<i>E. coli</i> extract added, time 0 min.			1.69 (0.675 mm)	0.01 (0.86 mm)
3	No extract added, time 60 min.			1.73 (0.67 mm)	0 (0.86 mm)

In Experiments 1 and 2 the cups contained 6 ml. of *Escherichia coli* extract; all cups contained 0.75 mm of  $\text{NaHCO}_3$ , 0.69 mm of  $\text{HC}^{13}\text{OONa}$ , 0.86 mm of Na pyruvate, and 0.25 mm of phosphate (pH 6.8) in a total volume of 18 ml. under an atmosphere of 90 per cent  $\text{N}_2$  and 10 per cent  $\text{CO}_2$ .

dismutation of pyruvate to acetate, carbon dioxide, and lactate, but the rate of this reaction is slow compared with that of the phosphoroclastic reaction.

The reaction was carried out by incubating the bacterial extract with the sodium salts of  $\text{HC}^{13}\text{OOH}$  and pyruvic acid. When the reaction was stopped after 60 minutes, 30 per cent of the pyruvic acid had been broken down. The reaction mixture was acidified with 5 ml. of 10 N sulfuric acid, and the carbon dioxide liberated from the sodium bicarbonate was collected in 2.5 N carbon dioxide-free alkali. The mixture was then largely freed of protein by centrifugation. Formic and acetic acids are almost completely volatile by steam distillation, whereas pyruvic acid is only about 60 per cent volatile. By steam distilling the centrifugate twice until 12 to 14 volumes have been collected, the formic and acetic acids were obtained in the distillate, whereas about two-thirds of the pyruvate was present in

the combined residues. An excess of ceric sulfate was added to the combined residues, and after 10 minutes at room temperature in a closed system, the excess was destroyed by addition of ferrous sulfate and the mixture boiled and aerated. The carbon dioxide liberated from the carboxyl group was collected in carbon dioxide-free alkali as before.

The pyruvic acid in the distillate was destroyed by ceric sulfate, the mixture filtered, redistilled, and HCOOH oxidized to carbon dioxide and water (Osburn *et al.* (12)).

Examination of the data in Table I reveals that the pyruvate-COOH and HCOOH were in equilibrium as shown by the equal  $C^{13}$  values. Theoretical equilibrium values for the two compounds as calculated from the amounts of the pyruvate and formate and excess  $C^{13}$  present show that

TABLE II  
*C<sup>13</sup> Values in Pyruvate-COOH by Yeast Carboxylase and by Ceric Sulfate*

Pyruvate dissimilated, mm.....	0.32
Excess $C^{13}$	atom per cent
HCOOH.....	0.94
Pyruvate-COOH.....	
Ceric sulfate.....	0.41
Yeast carboxylase.....	0.39*

The experimental cup contained 12 ml. of *Escherichia coli* extract, 1.5 mm of  $NaHCO_3$ , 0.8 mm of  $HC^{13}OONa$  ( $C^{13}$  excess = 2.37 atom per cent), 1 mm of phosphate (pH 6.8), and 2.3 mm of pyruvate in a total volume of 30 ml. The experiment was conducted for 45 minutes under 90 per cent  $N_2$  and 10 per cent  $CO_2$ .

\* Corrected for a small residual of normal  $CO_2$  present in the yeast preparation as determined by acidification.

both compounds should contain 0.79 excess  $C^{13}$  atom per cent. The validity of the separation and analytical methods and the enzymatic nature of the reaction are shown by the two controls listed in Table I (Experiments 2 and 3).

The very low excess  $C^{13}$  in the bicarbonate demonstrates the almost complete absence of hydrogenlyase in the bacterial extract. This fact has been verified by several experiments; also the preparation is completely unable to form carbon dioxide and hydrogen from formic acid. The absence of hydrogenlyase makes the data more conclusive. In the presence of hydrogenlyase, as shown by experiments with whole cells described later,  $C^{13}O_2$  can be fixed in pyruvate-COOH, but some doubt exists as to the path of fixation. Combination of a  $C_3$  compound + carbon dioxide compounds followed by conversion to a symmetrical molecule and reconversion to pyruvate could also result in  $C^{13}$  fixation in the pyruvate-COOH. How-

ever, since no  $C^{13}O_2$  was present in this experiment with extracts, such a mechanism could not play a part.

The concentration of  $C^{13}$  in the pyruvate-COOH obtained during one exchange experiment was determined biologically as well as chemically (Table II). Decarboxylation of the residual pyruvate by yeast carboxylase and by ceric sulfate gave comparable values. After removal of the carbon dioxide by acidification and the proteins by centrifugation, the fermentation mixture was steam-distilled three times. The combined residues of the three distillations were continuously extracted with ether for 24 hours. One portion of the extract containing the pyruvic acid was oxidized with ceric sulfate; the remainder was brought to pH 6.2 and the pyruvate decarboxylated by Lebedev juice. The carbon dioxide and aldehyde formed by the reaction were removed by a continuous passage of nitrogen through a series of towers containing sodium bisulfite, potassium permanganate, and carbon dioxide-free sodium hydroxide. The carbon dioxide so obtained has a  $C^{13}$  excess of 0.39 atom per cent as compared to 0.41 for the ceric sulfate.

#### *Effect of Phosphate on Exchange between Formate and Pyruvate*

Since the reaction under study in reality involves phosphorylation, an examination of the effect of inorganic phosphate on the rate of exchange between  $HC^{13}OOH$  and pyruvic acid was made. When an excess of phosphate was added to the extract, the rate of exchange was increased (Table III). Still more clearly the effect of phosphate appeared when experiments were performed with dialyzed extracts. The results recorded in Table IV were obtained with an *Escherichia coli* extract which had been dialyzed for 40 minutes against ice-cold water. The disappearance of pyruvate is little affected by the phosphate concentration in spite of the noticeable stimulation of the exchange reaction. Accumulation of acetyl phosphate is expected to stimulate the exchange since it reacts with  $HCOOH$ . Previously it has been shown (16) that the concentration of inorganic phosphate plays an important rôle in the accumulation of acetyl phosphate during pyruvate dissimilation. With very low concentrations no demonstrable acetyl phosphate may accumulate, whereas with high phosphate concentrations there was little tendency for the acetyl phosphate to dephosphorylate and it accumulated in considerable quantities (cf. Table III).

Already such amounts of acetyl phosphate as were accumulated at relatively low phosphate concentrations appeared sufficient to maintain rapid exchange (Table III). When a considerable excess of synthetic acetyl phosphate was added, no increase of turnover was observed. On the contrary, a decrease of exchange as well as of pyruvate dissimilation was caused. Partly this inhibitory effect persisted with solutions of decom-



posed acetyl phosphate and may be attributed to inhibitory impurities. A truly inhibitory action on the enzyme system by acetyl phosphate in higher concentration, however, is not improbable. The results of these experiments were rather erratic and the nature of the effect is under further investigation. Sodium acetyl phosphate obtained from the silver salt and

TABLE III

*Effect of Phosphate upon Exchange between  $\text{HC}^{13}\text{OOH}$  and  $\text{CH}_3\text{COCOOH}$  in Undialyzed Extract*

Experiment No.	Phosphate added	Pyruvate dissimilated	Acetyl phosphate formed	Excess $\text{C}^{13}$ , atom per cent	
				Pyruvate-COOH	HCOOH
	mm	mm	mm		
1		0.10	0.009	0.59	0.89
2	0.5	0.189	0.082	0.67	0.75
3	1.0	0.186	0.086	0.69	0.80

Each cup contained 7 ml. of undialyzed *Escherichia coli* extract, 0.09 mm of  $\text{Na}^+\text{HCO}_3^-$ , 0.64 mm of pyruvate, and 0.69 mm of HCOOH (excess  $\text{C}^{13} = 1.72$  atom per cent) in a total volume of 20 ml.; under 90 per cent nitrogen and 10 per cent carbon dioxide for 45 minutes at  $30.4^\circ$

TABLE IV

*Effect of Phosphate upon Exchange between  $\text{HC}^{13}\text{OOH}$  and  $\text{CH}_3\text{COCOOH}$  in Dialyzed Extracts*

Experiment No.	Added phosphate	Pyruvate dissimilated	Excess $\text{C}^{13}$ , atom per cent	
			Pyruvate-COOH	HCOOH
	mm	mm		
1		0.132	0.12	1.51
2	0.2	0.14	0.32	1.21
3	0.4	0.156	0.40	1.14

Each flask contained 6.0 ml. of dialyzed *Escherichia coli* juice, 0.6 mm of  $\text{NaHCO}_3$ , 0.6 mm of pyruvate, and approximately 0.3 mm of  $\text{HC}^{13}\text{OONa}$  ( $\text{C}^{13} = 2.37$  atom per cent) in a total volume of 16 ml. The flasks were incubated for 40 minutes under 90 per cent nitrogen and 10 per cent carbon dioxide.

lithium acetyl phosphate obtained by 2-fold reprecipitation with alcohol have given similar results so far.

The pyruvate-COOH and HCOOH were determined by ceric sulfate and  $\text{HgO}$  oxidation respectively after separation as described.

#### *Exchange between $\text{CH}_3\text{C}^{13}\text{OOH}$ and Pyruvic Acid*

To establish more clearly the nature of the  $\text{C}_2 + \text{C}_1$  addition, experiments were conducted with  $\text{CH}_3\text{C}^{13}\text{OOH}$ . If the fixation reaction occurs, the

carbonyl group of the residual pyruvate should contain an excess of  $C^{13}$ . Analysis of the pyruvic acid revealed that a significant excess of  $C^{13}$ , 0.14 atom per cent, was fixed in this position (Table V). In Experiment 2, 0.11 atom per cent excess  $C^{13}$  was present in the  $CH_3CO-$  fraction, which gives a value of 0.22 atom per cent for the carbonyl group if all of the excess is in this portion of the molecule. Significant fixation occurred, however, only with added adenylyl pyrophosphate. The exchange between  $CH_3C^{13}OOH$  and pyruvic acid is of necessity slower than that between

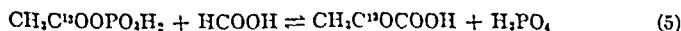
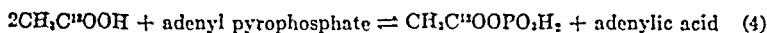
TABLE V  
Fixation of  $CH_3C^{13}OOH$  in Pyruvic Acid by *Escherichia coli* Extract

Compound	Experiment 1		Experiment 2	
	mm	$C^{13}$ excess	mm	$C^{13}$ excess
		atom per cent		atom per cent
$CH_3COCOOH$ . . . . .	0.45		0.475	
$CH_3CO-$ . . . . .				0.11 (Persulfate oxidation)
$-CO-$ . . . . .		0.14		0.22 (Calculated)
$CH_3COOH$ . . . . .	0.90	1.79	1.55	1.78
$-COOH$ (calculated) . . . . .		3.58		3.56
$HCOOH$ . . . . .	0.57	0.01		

Experiment 1—8.0 ml. of *Escherichia coli* extract, 0.6 mm of  $NaHCO_3$ , 1.0 mm of phosphate (pH 6.8), 0.72 mm of  $CH_3C^{13}OOH$  ( $C^{13}$  of  $-COOH = 5.07$  atom per cent), 0.4 mm of  $HCOONa$ , 0.65 mm of pyruvate, and 10 mg. of adenylyl pyrophosphate in a total volume of 22 ml. Time, 75 minutes; atmosphere, 90 per cent  $N_2$  and 10 per cent  $CO_2$ .

Experiment 2—12 ml. of *Escherichia coli* extract, 1.5 mm of  $NaHCO_3$ , 1.0 mm of phosphate (pH 6.8), 1.16 mm of  $CH_3C^{13}OOH$  ( $C^{13}$  of  $-COOH = 5.07$  atom per cent), 0.9 mm of pyruvate, and 20 mg. of adenylyl pyrophosphate in a total volume of 32 ml. Time, 75 minutes; atmosphere, 90 per cent  $N_2$  and 10 per cent  $CO_2$ .

$HC^{13}OOH$  and pyruvic acid, since two reactions rather than one are involved in the former case. The reversibility of Reaction 4 has been shown by Lipmann (7).



Reaction 5 completes the exchange of  $CH_3C^{13}OOH$  and pyruvic acid. Since adenylyl pyrophosphate is an integral part of Reaction 4, its presence is necessary for the exchange reaction. The effect of adenylyl pyrophosphate is shown in Table VI.

The fermentation liquid was twice steam-distilled and the combined residues extracted with ether. The extracted pyruvic acid was decomposed by yeast carboxylase. The  $CH_3CHO$  trapped in sodium bisulfite

solution was decomposed by the iodoform reaction. The  $-\text{CHO}$  group, representing the carbonyl group of the pyruvate molecule, was converted to  $\text{HCOOH}$  and the  $\text{CH}_3-$  group to  $\text{CHI}_3$ . The iodoform was filtered off; the  $\text{HCOOH}$  was recovered by steam distillation and then oxidized by  $\text{HgO}$ . In another experiment pyruvate was converted to acetate and carbon dioxide by ceric sulfate oxidation and the acetate or  $\text{CH}_3\text{CO}-$  portion of the pyruvate was oxidized to carbon dioxide by persulfate (12).

The distillate containing acetic and formic acids was again twice redistilled and was then free of pyruvic acid. Formic acid was removed by

TABLE VI

*Effect of Adenyl Pyrophosphate on Fixation of  $\text{CH}_3\text{C}^{13}\text{OOH}$  in Pyruvic Acid*

Experiment No.	Adenyl pyrophosphate added	$\text{C}^{13}$ excess in $-\text{CO}-$ of pyruvate*
	mg. per ml.	atom per cent
1	0	0.05
2	0	0.05
3	0	0.02
3a	0.45	0.14
4	0.62	0.22

Experiments 3 and 3a are parallel experiments with the same sample of extract. The others were carried out with different extracts. Otherwise the experiments were carried out in approximately the same manner. See Table V for greater detail as to the set-up; Experiments 3a and 4 are the same as Experiments 1 and 2 in Table V.

\* Values determined directly as  $-\text{CO}-$  in Experiments 2, 3, and 3a and calculated from the  $\text{C}^{13}$  content of  $\text{CH}_3\text{CO}-$  in Experiments 1 and 4.

$\text{HgO}$  oxidation and after filtration and redistillation the acetate was oxidized to carbon dioxide by persulfate.

#### *Fixation of $\text{C}^{13}\text{O}_2$ in Pyruvic Acid by Viable *Escherichia coli**

Although the cell-free preparation from *Escherichia coli* contains no hydrogenlyase, whole cell suspensions of the organism are able to convert  $\text{C}^{13}\text{O}_2$  first to  $\text{HC}^{13}\text{OOH}$  and then to  $\text{CH}_3\text{COC}^{13}\text{OOH}$  (Reactions 2 and 3). Results of such an experiment are shown in Table VII. The experiment was conducted by placing  $\text{NaHC}^{13}\text{O}_3$  and formic acid in one side arm and the pyruvic acid in the second side arm. After preliminary equilibration, the formic acid buffer-cell mixture was allowed to incubate for 60 minutes, when the pyruvic acid was added and the reaction allowed to proceed for an additional 40 minutes.

The  $-\text{COOH}$  of the residual pyruvate contained an excess of 0.37 atom per cent  $\text{C}^{13}$ , and the  $\text{HCOOH}$  contained 1.99 atom per cent excess  $\text{C}^{13}$ . It is possible that some of the  $\text{C}^{13}\text{O}_2$  in the pyruvate may have been fixed

by a route involving  $C_3 + C_1$  addition. *Escherichia coli* grown on a carbohydrate medium, however, does not form appreciable quantities of succinic acid, and it is doubtful whether the  $C_3 + C_1$  addition plays an appreciable rôle in these cells. In any case the path for  $C^{13}O_2$  fixation in pyruvate by hydrogenlyase and the phosphoroclastic reactions has been definitely established in these cells, whereas the other has not.

In general, the reaction mixture was treated by methods already discussed.

TABLE VII

*Fixation of  $C^{13}O_2$  in Pyruvic Acid by Cell Suspension of *Escherichia coli**

Compounds	Excess $C^{13}$	
	mm	atom per cent
$CH_3COCOOH$ .....	0.23	
$CH_3CO-$ .....		0
$-COOH$ .....		0.37
$HCOOH$ .....	2.05	1.99
$CH_3COOH$ .....	0.73	0.01
$NaHCO_3$ .....		2.44

The manometric flask contained 7 ml. of a 15 per cent suspension of *E. coli*, 1.8 mm of  $NaHC^{13}O_3$  ( $C^{13} = 4.75$  atom per cent), 1.2 mm of pyruvate; 1.4 mm of  $HCOONa$ , and 1.5 mm of phosphate (pH 6.6) in a total volume of 30 ml. The experiment was conducted under  $H_2$  for a total time of 100 minutes. Approximately 0.97 mm of pyruvate was fermented.

#### DISCUSSION

The phosphoroclastic reaction is limited to a number of bacteria of the family Bacteriaceae. However, the other similar reactions involving the formation of acetic acid and carbon dioxide from pyruvate are well known in other bacteria and in tissue. These reactions differ from the phosphoroclastic mainly with regard to the reduced substance formed. If these reactions give rise to acetyl phosphate rather than acetic acid, these reactions also may prove reversible.

Koepsell, Johnson, and Meek (2) have reported that an enzyme system obtained from *Clostridium butylicum* forms acetyl phosphate, carbon dioxide, and hydrogen. Hydrogen inhibits the reaction and for this and other reasons reversibility is suggested (9). As strong evidence for the reversibility of this reaction, Brown, Wood, and Werkman<sup>2</sup> have found a large excess of  $C^{13}$  in the  $COOH$  group of lactic acid formed by *Clostridium butylicum* from glucose in the presence of  $C^{13}O_2$ . The fixation seems unlikely to have taken place by  $C_3 + C_1$  addition, since the investigation

<sup>2</sup> Brown, R. W., Wood, H. G., and Werkman, C. H., unpublished.

revealed that this organism shows little activity on the  $C_4$ -dicarboxylic acids. Slade *et al.* (14) also report fixation of  $C^{13}O_2$  in lactate- $COOH$  by *Cl. welchii* and *Cl. acetobutylicum* in spite of the fact that no  $C_4$ -dicarboxylic acids were formed during the fermentation. A  $C_2 + C_1$  condensation can easily account for this fixation, although direct proof is lacking.

The dismutation of pyruvic acid to lactic and acetic acids and carbon dioxide has been shown in bacteria (11, 4) and in tissue (6). By analogy it seems possible that this reaction may also prove reversible.

Certain data already incorporated in the literature may need further examination. Slade and Werkman (13) found that *Aerobacter indologenes* acting upon  $CH_3C^{13}OOH$  or  $C^{13}H_3C^{13}OOH$  in the presence of glucose forms 2,3-butylene glycol containing  $C^{13}$  in the carbinol carbons in the case of  $CH_3C^{13}OOH$  and in all carbons in the addition of  $C^{13}H_3C^{13}OOH$ . Slade and Werkman (13) suggested that a conversion of the  $C^{13}$  acetate to acetaldehyde followed by condensation could account for the  $C^{13}$  content of 2,3-butylene glycol. An alternate explanation is now possible by which acetate is converted to pyruvic acid and the latter converted to 2,3-butylene glycol without the necessary intervention of acetaldehyde.

#### SUMMARY

1. When pyruvic acid is dissimilated by *Escherichia coli* extract in the presence of  $HC^{13}OOH$ , the residual pyruvate is shown to contain excess  $C^{13}$  in the carboxyl group, which is evidence for reversibility of the pyruvate split.

2. Addition of inorganic phosphate to dialyzed *Escherichia coli* extract increases the rate of exchange.

3. When pyruvic acid is broken down in the presence of  $CH_3C^{13}OOH$  and of adenylyl pyrophosphate, the residual pyruvate contains excess of  $C^{13}$  in the carbonyl group.

4. Intact cells of *Escherichia coli* acting upon pyruvic acid in the presence of  $NaHC^{13}O_3$  fix  $C^{13}$  in the carboxyl group of pyruvic acid.

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alcoholic hydrochloric acid, in a 90 per cent acetone medium containing naphthyl red as an indicator. At the point where the hydrogen ion concentration is sufficient to cause the indicator to change from the yellow to the red range (pH 5 to 3.7 in aqueous solution), the amount of light transmitted through the cell decreases abruptly, and this decrease is registered quantitatively by the galvanometer.

The instrumental assembly is illustrated in Figs. 2 and 3.<sup>1</sup> A 110 volt alternating current passes through a voltage stabilizer, the output of which is stepped up by means of a transformer to the voltage required for the operation of a mercury vapor lamp. The light emanating from the mercury vapor lamp passes through the filter, then through a narrow slit,  $1.5 \times 2.5$

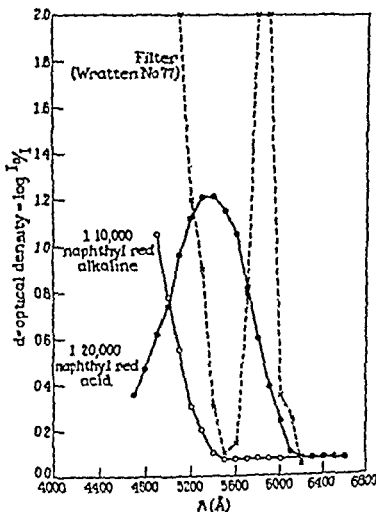


FIG. 1. Relation of absorption spectrum of Wratten Filter 77 to absorption spectra of naphthyl red in acid and alkaline ranges.

mm. in size, in the micro cuvette holder. This slit is so arranged that the light coming through it travels parallel to one side of the micro cell, while the electromagnetic stirrer operates close to the other side of the cell. In this way the stirrer does not interrupt the path of light during the titration.

The cuvette holder itself is made from a solid bakelite block,<sup>2</sup> with holes drilled so as to permit the insertion of the micro cell and the passage of the light beam to the photronic cell. The core of the electromagnet is arranged so as to touch one side of the micro cell. The multiple mirror galvanometer

<sup>1</sup> A Pfaltz and Bauer fluorophotometer model A was used.

<sup>2</sup> The authors are indebted to Mr. Joseph Bloom of the Rockefeller Institute for the construction of the cuvette holder.

used has a sensitivity of  $2 \times 10^{-9}$  ampere per mm. and a resistance of 640 ohms.

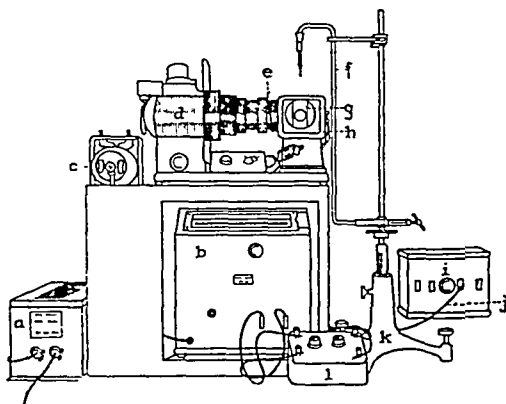


FIG. 2. Instrumental assembly. *a*, voltage stabilizer; *b*, multiple mirror galvanometer; *c*, micro switch circuit breaker; *d*, mercury arc lamp; *e*, filter; *f*, micro burette; *g*, cuvette housing; *h*, photonic cell; *i*, transformer; *j*, connection to voltage stabilizer; *k*, base with rack and pinion; *l*, variable shunt resistance box.

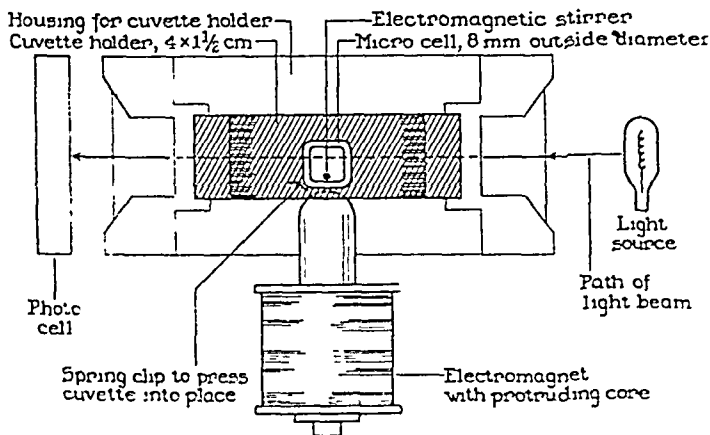


FIG. 3. Detailed diagram of micro cuvette in holder, showing relationship of electromagnetic stirrer to light beam.

The micro burette is of a mercury screw design with a fineness about twice that of the micro burette previously described by Linderstrøm-Lang and Holter (6). The burette is mounted on a firm base equipped with a rack and pinion, so that the tip may be inserted into the titrating



solution or elevated out of the way. A micro switch current interrupter provides the means for operating the stirrer at the rate of 100 to 120 times a minute, found desirable for maintenance of adequate stirring. The stirrers themselves may be made by sealing 1 to 2 mm. lengths of No. 24 annealed steel wire in thin walled capillary tubing. Provided its total length is under 3 mm., the stirrer does not interrupt the light beam traversing the cell.

The micro cells are made from square 6 × 6 mm. ungraduated hemometer tubing, 30 mm. tall. They are used both for reaction vessels and titrating vessels. In a single series of titrations, only cells whose light transmission was the same within 1 per cent were used.

### Results

*Determination of End-Point*—As a test of the precision of the method, the following experiment was carried out: 5.60 c.mm. samples of a 0.10 M solution of leucylglycine were pipetted into a series of six vessels by means of a Levy (7) semiautomatic pipette. 300 c.mm. of acetone containing 0.02 per cent naphthyl red were added to each vessel just prior to titration against 0.0507 N hydrochloric acid in 90 per cent ethanol. The vessel was set in place and stirring begun, the tip of the burette dipped 1 to 2 mm. below the surface of the titrating medium, the galvanometer set at 100 per cent transmission of the incident light, and the titration started. At the point where the galvanometer registered 70 per cent transmission, the rate of decrease in light transmission per c.mm. of hydrochloric acid was maximal, and this point was chosen as the end-point for the titration.

A single titration required approximately 5 minutes. The theoretical titration value was 11.05 c.mm. of hydrochloric acid and the actual arithmetic mean titration value was 11.05 c.mm. The series of readings was as follows: 10.97, 11.10, 11.01, 11.15, 10.97, and 11.13 c.mm. of hydrochloric acid. The greatest deviation from the mean was 0.9 per cent and the standard deviation was 0.08 c.mm. When the titration figures are of this order of magnitude, the titration error is therefore likely to be less than 1 per cent. The pipetting error is also included in these calculations.

As a further test of the sensitivity of the method, samples of 5.65 c.mm. of a 0.005 M leucylglycine solution were pipetted into a series of vessels, which, after the addition of acetone and indicator, were then titrated, 0.011 N alcoholic hydrochloric acid being used. The consecutive readings were 2.60, 2.63, 2.59, 2.64, 2.62, and 2.57 c.mm. of hydrochloric acid. The arithmetic mean of the titration values was 2.61. The calculated titration value was 2.57. The greatest deviation from the arithmetic mean was 1.5 per cent and the standard deviation was 0.03. By this method, then,  $2.8 \times 10^{-3}$  mole of leucylglycine can be titrated with considerable accuracy.

*Enzymatic Experiments*—As a test of the method in actual practice, the following enzymatic experiment was performed: 5.65 c.mm. of solution containing 0.10 M benzoyl-L-argininamide and 0.02 M secondary sodium citrate buffer were pipetted into a reaction vessel followed by 5.65 c.mm. of solution of purified papain containing 0.066 mg. of protein nitrogen per cc. and 0.02 M cysteine. The mixture was stirred electromagnetically, and the open vessel was incubated at 40° in a nitrogen chamber. The pH of the reaction was 5.0. Control vessels were set up in the same manner as the incubated ones, but titration was performed immediately. The results of the enzymatic experiment are given in Table I.

The nitrogen chamber mentioned above consists of a 250 cc. drying tower, equipped with a rack which holds the micro vessels in place, with their bases immersed in 0.09 M sodium chloride. Nitrogen is bubbled through a wash bottle filled with 0.09 M sodium chloride, then into the

TABLE I  
*Hydrolysis of Benzoylargininamide by Cysteine-Papain*

Time	Amount of HCl required*	Extent of hydrolysis	$K \times 10^{\dagger}$
min.	c.mm.	per cent	
60	3.28	29	2.49
80	4.15	37	2.49
95	4.86	43	2.57
120	5.79	51	2.60
165	7.12	63	2.62

\* Incubated sample minus control.

$$\dagger K = \frac{1}{t} \log \frac{a}{a-x}.$$

nitrogen chamber. Both bottles are immersed in a constant temperature water bath. The micro vessels are thus incubated in an atmosphere of nitrogen, and evaporation from the vessels is avoided during the incubating time.

*Influence of Turbidity*—In conducting a photometric titration, the problem of dealing with turbidities in the titrating medium is an important one. It was found that the 90 per cent or 95 per cent acetone medium used precipitated the enzyme protein, giving rise to turbidity in the titration medium. Since this turbidity was constant throughout the titration, however, it did not affect the rate of change of the indicator color. The galvanometer showed a smooth downward deflection at the end-point, free from undesirable small fluctuations. If, however, the protein concentration, and consequently the protein precipitate, was 10 times that used in our experiments, then undesirable fluctuations in the galvanometer

were observed when this precipitate was stirred during the course of a titration.

A more serious difficulty is introduced by crystallization of the substrate or the buffer out of the titration medium. As Linderström-Lang (5) points out, this error usually may be avoided if the greater part of the hydrochloric acid required to reach the end-point is added to the test solution prior to the acetone.

There is another advantage to adding, prior to titration, most of the acid necessary to reach the end-point. Addition of hydrochloric acid to the titration medium results in a gradual decrease in the concentration of indicator and of turbidity, with a consequent decrease in the optical den-

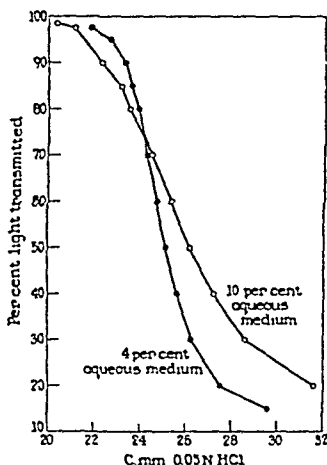


FIG. 4

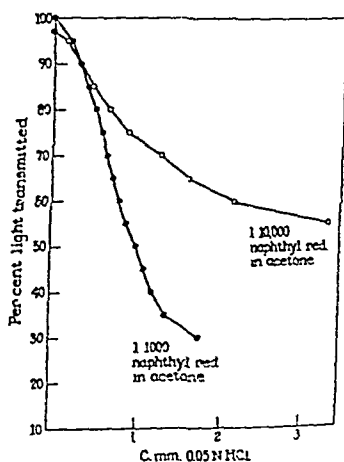


FIG. 5

FIG. 4. Effect of concentration of aqueous medium on sharpness of end-point.

FIG. 5. Effect of indicator concentration on light transmission at end-point.

sity. This source of potential error was further minimized by using a concentration of hydrochloric acid such that the volume of the titration medium was increased only to a small degree during the titration (indicated by a slight upward deflection of the galvanometer before the end-point was reached). The control titration cancels out the larger part of this error. The indicator, in the appropriate concentration, might conceivably be included in the hydrochloric acid being titrated in, in order to reduce this error still further.

*Factors Influencing Indicator Color Change*—Micro titration with a photonic cell makes it convenient to study in detail the rate and extent of change of the indicator color at its end-point. In general, the greater the

concentration of the acetone and the less the concentration of water in the titration medium, the sharper does the end-point become. It is therefore of advantage to employ the highest concentration of acetone which can be used without causing the substrate or buffer to crystallize out. Fig. 4 shows that as the aqueous concentration of the titration medium is decreased below 10 per cent the end-point becomes more abrupt, making possible more accurate titration. The composition of the test solution, 11.15 c.mm., was as follows: 0.05 M carbobenzoxyglycyl-L-phenylalanine, 0.02 M secondary sodium citrate buffer, 0.01 M cysteine, swine kidney cathepsin, and 0.22 mg. of protein nitrogen per cc. of test solution.

The greater the concentration of the indicator in the titration medium, the greater is the decrease in the absolute amount of transmitted light caused by the color change of the indicator (*cf.* Fig. 5). It is therefore desirable to use a high concentration of indicator in the titration medium.

If the split-products of an enzymatic reaction are better buffers than the original substrate in the pH range of color change of the indicator, the indicator will then change color less sharply when the incubated samples are titrated than it does when the control samples are titrated. The slopes of the end-point curves will differ in the two cases, and an error will be introduced if a certain shade of color or per cent of light transmitted is chosen as an end-point. The magnitude of this error will depend on the degree to which the slopes of the curves in question differ.

An illustration of this buffering effect of the split-products is provided by the action of carboxypeptidase upon the hydrolysis of carbobenzoxyglycyl-L-phenylalanine on acidimetric titration in an acetone medium, with naphthyl red as an indicator. In these circumstances, the phenylalanine split off is a better buffer than the carbobenzoxyglycyl-L-phenylalanine. Fig. 6 shows the effect on the end-point of altering the concentrations of these two compounds in a known manner in a 10 per cent aqueous medium. As the concentration of carbobenzoxyglycyl-L-phenylalanine is decreased and the concentration of phenylalanine is increased (as would occur in the enzymatic hydrolysis of carbobenzoxyglycyl-L-phenylalanine), the slopes of the titration end-point curves become flatter. 11.15 c.mm. of substrate mixture, containing 0.03 M phosphate buffer at pH 7.44, were used in all cases. There were 330 c.mm. of titration medium, containing 0.1 per cent naphthyl red, and consisting of 90 per cent acetone and 10 per cent aqueous phases.

As the concentration of water in the titration medium is decreased below 10 per cent, and the concentration of acetone is correspondingly increased, the ionization of substrate and split-products may be depressed sufficiently, in the case of carbobenzoxyglycyl-L-phenylalanine and phenylalanine, to make the error introduced by the buffering effect of the split-products a

negligible one. Fig. 7 shows the titration curves obtained in an enzymatic experiment in which carbobenzoxyglycyl-*L*-phenylalanine was split by swine kidney cathepsin, and the titration of samples carried out in a 4 per cent aqueous medium. The slope of all the curves here is essentially the

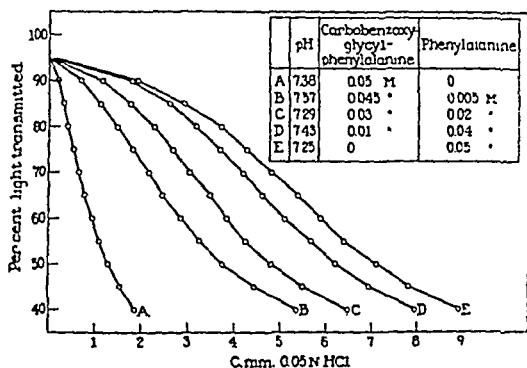


FIG. 6. Effect on slope of titration curve of increasing the concentration of one of the split-products. The abscissa indicates only the amount of hydrochloric acid added after the indicator color change.

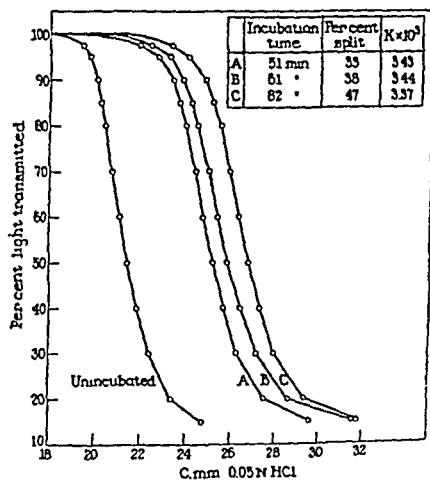


FIG. 7. Titration end-point curves in an enzymatic experiment

same, and the reaction velocity constants calculated from the data are remarkably close. 11.15 c.mm. samples of test solution were pipetted into individual vessels, which were incubated at 40° in nitrogen for varying times before being removed and titrated in a 96 per cent acetone, 4 per cent

aqueous medium containing 0.1 per cent naphthyl red. The test solution was composed of carbobenzoxyglycyl-L-phenylalanine 0.05 M, secondary sodium citrate buffer 0.02 M, cysteine 0.01 M, and swine kidney cathepsin 0.22 mg. of protein nitrogen per cc. of test solution. The pH of the reaction was 5.2 in all cases.

#### SUMMARY

A micro titration method for the measurement of enzymatic proteolysis is described in which a photometric determination of the end-point is substituted for a visual one. The photometric titration may be adapted without difficulty to the semimicro scale of the usual determinations of enzymatic cleavages of peptides, which hitherto have been performed by visual end-point determinations.

The objective photometric titration has revealed a possible source of error which may interfere with the titration when one of the split-products of the enzymatic cleavage buffers the system against pH changes more effectively than does the original peptide.

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# ON THE HYPOGLYCEMIC ACTION OF ALLOXAN

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Jacobs (1) observed that an intravenous injection of alloxan in rabbits produced a fall in the blood sugar level leading to hypoglycemic convulsions. This was confirmed by Dunn and McLetchie (2), Bailey and Bailey (3), Hughes *et al.* (4), Brunschwig *et al.* (5), Goldner and Gomori (6), and Kennedy and Lukens (7). If the animals were given intravenous injections of glucose so as to counteract the effect of hypoglycemia, they survived and developed hyperglycemia and glycosuria. The cause of this transient hypoglycemia has been differently explained. Dunn and McLetchie (2) think that hypoglycemia is due to the stimulating action of alloxan on the islands of Langerhans, whereby an increased quantity of insulin is released into the circulation. The cells of the islands of Langerhans become necrosed, being exhausted from overwork, leading to the symptoms of diabetes mellitus. Hughes *et al.* (4), on the other hand, consider that this fall in the blood sugar level is due to the liberation of preformed insulin from the necrosed cells of the islands of Langerhans and that there is no overproduction of insulin. They came to this conclusion because they observed that the hypoglycemic action of alloxan could be simulated by the injection, in the form of protamine zinc insulin, of that amount of extractable insulin which is known to be present in the pancreas of a normal rabbit. However, they admit that this evidence is not conclusive. Ridout *et al.* (8) studied the insulin content of the pancreas of dogs killed at different times after the injection of alloxan. They did not notice any significant change in the insulin content of the pancreas up to 8 hours after the injection, by which time the hypoglycemia becomes most marked in dogs and rabbits (7, 8). They therefore consider that the hypoglycemia is not due to overproduction of insulin. This observation, however, may not be contrary to the overproduction theory, because it might be argued that insulin as soon as formed by overstimulation is released into the circulation and consequently there is no increase in the insulin content of the pancreas. Ridout *et al.* (8) also reported that alloxan could not produce hypoglycemia in depancreatized dogs and in dogs made diabetic by previous injection of alloxan. This observation was also confirmed by Kennedy and Lukens

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(7) in rabbits. These investigators think that this is also evidence against the overproduction theory. This argument does not seem to be valid, owing to the fact that if there is no normal islet tissue left, by previous injection of alloxan or by the removal of the pancreas, alloxan cannot produce this action.

In order to elucidate the mechanism of alloxan hypoglycemia, I partially pancreatectomized rabbits and studied the blood sugar curve after injection of alloxan. The amount of pancreatic tissue left after operation was just sufficient to maintain the normal fasting blood sugar level. The idea was that if the action of alloxan is that of stimulation, so that the islet

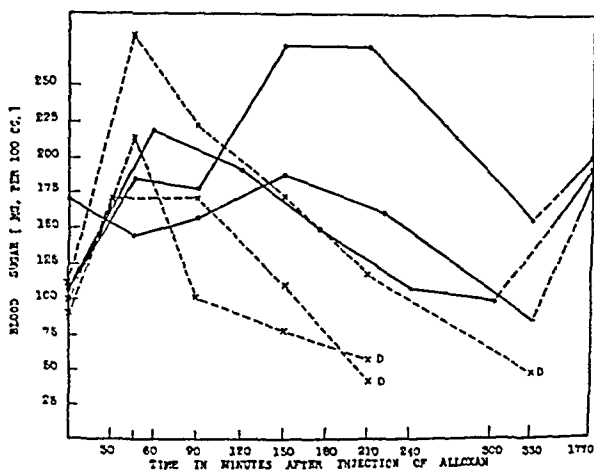


FIG. 1. Blood sugar curves of rabbits injected with alloxan. The solid curves represent results with animals partially pancreatectomized; the dash curves, animals with the pancreas intact.

cells are necrosed due to overactivity, the remnant of the pancreas in the partially pancreatectomized rabbits would release enough insulin to cause hypoglycemic convulsions in these animals. On the other hand, if alloxan only releases the preformed insulin by its necrosing action on the islets, the blood sugar will be lowered to a lesser extent because the amount of insulin released from the pancreatic remnant will be much less. The experimental results obtained are presented in this paper.

#### EXPERIMENTAL

Nine healthy male Himalayan rabbits of weights varying between 800 and 1450 gm. were used. All the animals were fasted overnight. Six were anesthetized with ether and about a half of the pancreas was removed by a

median longitudinal abdominal incision. 3 hours after the operation three of the partially pancreatectomized rabbits and three others with intact pancreas were given a single intravenous injection of a 10 per cent solution of alloxan (200 mg. per kilo). Samples of blood were drawn at intervals from the ear vein. Blood sugar was estimated according to the method of Hagedorn and Jensen (9). The blood sugar curves are shown in Fig. 1.

Three partially pancreatectomized rabbits not injected with alloxan were placed in separate metabolism cages and their urine was collected under toluene. The fasting blood sugar level was estimated next morning and the urine was examined for the presence of sugar with Benedict's reagent. The blood sugar values were 100, 110, and 116 mg. per 100 cc.; the urine showed a trace of sugar.

### *Results*

In three of the rabbits with intact pancreas, the blood sugar fell rapidly, after a transient rise, following the injection of alloxan and all the animals died of hypoglycemic convulsions. The three partially pancreatectomized rabbits similarly treated not only did not develop marked hypoglycemia but also survived and showed hyperglycemia and glycosuria on the following day (Fig. 1). The three partially pancreatectomized rabbits not injected with alloxan excreted only traces of sugar, while the fasting blood sugar values were normal.

### DISCUSSION

Alloxan could not produce hypoglycemic convulsions in the partially pancreatectomized rabbits. The amount of pancreatic tissue left in these rabbits was just sufficient to maintain the normal fasting blood sugar level. If the injection of alloxan produced stimulation of the pancreatic remnant to such an extent as to cause death of the islet cells as a result of overwork, sufficient insulin should have been liberated to produce marked hypoglycemia. This, however, was not observed in our experiments. The slight hypoglycemia observed in the alloxan-treated, partially pancreatectomized rabbits appears to be due to the release of a smaller amount of preformed insulin from the necrosed islets of the pancreatic remnant into the circulation. Thus the hypoglycemic action of insulin seems not to be due to the overproduction of insulin. It is, however, to be noted that the hypoglycemic action of alloxan is not a constant phenomenon in rhesus monkeys (10).

### SUMMARY

1. Hypoglycemic convulsions were not observed when alloxan (200 mg. per kilo) was injected intravenously into three partially pancreatectomized rabbits.

2. All the three animals survived and developed hyperglycemia and glycosuria on the following day.

3. Three normal rabbits with the pancreas intact died of hypoglycemic convulsions within varying periods after the intravenous injection of alloxan.

4. The cause of the alloxan hypoglycemia is suggested to be due to the release of preformed insulin from the necrosed islets and not to stimulation of the islet tissue.

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# GROWTH INHIBITION BY ANALOGUES OF PANTOTHENIC ACID. PANTOTHENYL ALCOHOL AND RELATED COMPOUNDS

By ESMOND E. SNELL AND WILLIAM SHIVE

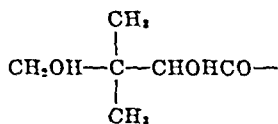
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Several compounds have been synthesized which are rather closely related in structure to pantothenic acid (1-9). The physiological properties of such compounds differ greatly. Some possess growth-promoting action similar to that of pantothenic acid itself; others are physiologically inert; and others inhibit growth. Of the former group, "hydroxypantothenic acid" (N-( $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethylolbutyryl)- $\beta$ -alanine (1-3)) and N-( $\alpha$ -hydroxy- $\beta$ -methyl- $\beta$ -methylolvaleryl)- $\beta$ -alanine (3) are most active, and possess growth-promoting activity from 2 to 25 per cent that of pantothenic acid, both for animals and for lactic acid bacteria. The most active member of the latter group so far known is pantoyltaurine<sup>1</sup> (4-6), the sulfonic acid analogue of pantothenic acid.

Recently, Pfaltz (10) reported that  $\alpha$ , $\gamma$ -dihydroxy-N-(3-hydroxypropyl)- $\beta$ , $\beta$ -dimethylbutyramide (N-pantoyl-3-propanolamine, pantotheryl alcohol) was as effective as pantothenic acid in preventing achromotrichia in rats. Presumably, this activity resulted from oxidation of the compound to pantothenic acid *in vivo*, but it appeared desirable to check activity of this compound against a number of other organisms. Yeast and various bacteria were used for this purpose. The product could not replace pantothenic acid for any of the microorganisms tested; in some cases it inhibited the growth ordinarily induced by pantothenic acid. This inhibition was competitive in nature; *i.e.*, it became apparent only when the *ratio* of pantotheryl alcohol to pantothenic acid exceeded a certain value, and was independent of the absolute amount of inhibitor added over a wide range of concentrations. A number of other compounds closely related to pantotheryl alcohol exhibited similar inhibitory properties, but to a lesser degree. Details of these findings are presented below.

<sup>1</sup> Throughout this paper, the simplified nomenclature adopted by Barnett and Robinson (5) will be used. In this system the name "pantoyl" is used for the  $\alpha$ , $\gamma$ -dihydroxy- $\beta$ , $\beta$ -dimethylbutyryl radical



## EXPERIMENTAL

*Pantothenyl Alcohol (N-Pantoyl-3-propanolamine)*—3-Propanolamine was prepared by the hydrogenation of  $\beta$ -hydroxypropionitrile (11) in dioxane solution in the presence of Raney's nickel catalyst under a pressure of 2000 pounds per sq. in. Addition of 0.698 gm. of the propanolamine to 1.20 gm. of freshly distilled  $dl$ - $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethylbutyrolactone resulted in a solution which evolved heat and increased in viscosity on standing to such an extent that it was necessary to heat the solution at  $110^\circ$  for 2 hours with occasional agitation in order to complete the reaction. The yield of pantothenyl alcohol, determined by amino nitrogen analyses before and after hydrolysis with hydrochloric acid, was 80 per cent of the theoretical.

Preparations utilizing optically active lactones were accomplished in the same manner; thus, 0.75 gm. of propanolamine and 1.31 gm. of (+)- $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethylbutyrolactone gave a 79 per cent yield of the alcohol, while 0.74 gm. of the propanolamine and 1.30 gm. of the (-)-lactone yielded 83 per cent of the theoretical.

A sample of the  $dl$ -pantothenyl alcohol was heated at  $110^\circ$  under high vacuum for 2 hours in order to remove unchanged intermediates. The purity of this sample was 88 per cent, as determined above.\*

*dl-N-Pantoylethanolamine*\*—Reaction of 0.594 gm. of freshly distilled ethanolamine and 1.259 gm. of  $dl$ - $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethylbutyrolactone was carried out in the manner described for pantothenyl alcohol. The yield determined similarly amounted to 81 per cent of the theoretical. After being heated for 2 hours at  $110^\circ$  in high vacuum, the product had a purity of 88 per cent.

*dl-N-Pantoyl-n-propylamine*—Upon addition of 0.72 gm. of *n*-propylamine to 1.31 gm. of  $dl$ - $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethylbutyrolactone, the mixture became warm. It was heated with occasional stirring for 2 hours at  $110^\circ$ . The yield was better than 90 per cent of the theoretical, based upon the lactone. The reaction mixture was heated for 2 hours under reduced pressure to remove unchanged propylamine. The viscous product showed little tendency to crystallize, but a small sample crystallized from petroleum ether-benzene after standing overnight at a low temperature. The remaining product was then crystallized from ether after first being seeded with a small amount of the crystalline material. Recrystallization from ether gave colorless octahedral prisms, m.p.  $45$ – $47^\circ$ .

*Analysis*— $C_8H_{13}O_2N$ . Calculated, N, 7.40; found, N, 7.42

\* By the procedures given, these compounds are obtained as viscous syrups which are very difficult to purify. Since preparation of these compounds has been described previously (10), and since the impurities present (chiefly unchanged lactone and amine) do not affect the physiological results, no extended effort was made to isolate the pure compounds. The dried condensation mixtures, containing about 90 per cent of the compound in question, were used in the experiments with micro-organisms.

*dl-N-Pantoylallylamine*—By the same process described for the *N*-propylamide, the allylamide was prepared from 0.76 gm. of allylamine and 1.30 gm. of the *dl*-lactone. The conversion based on the lactone was 95 per cent of the theoretical. In order to obtain a crystalline product, it was necessary to distill molecularly a small sample under a pressure of  $10^{-6}$  mm. at a temperature of 80–100°. The distillate solidified on standing overnight and a few crystals of this product added to the original crude product and to the residue from distillation induced crystallization. Recrystallization from ether gave colorless prisms, m.p. 42–44°.

*Analysis*— $C_8H_{17}O_2N$ . Calculated, N, 7.48; found, N, 7.46

*dl-N-Pantoylethylamine*—By heating a several fold excess of freshly distilled anhydrous ethylamine with 1.30 gm. of the *dl*-lactone in a sealed tube at 100° for 2 hours, the lactone was almost totally converted to the amide. After the excess ethylamine was removed by heating to 100° under reduced pressure, the product crystallized. On recrystallization from ether containing a few drops of ethanol, the compound separated slowly as colorless prisms, m.p. 64–66°.

*Analysis*— $C_8H_{17}O_2N$ . Calculated, N, 7.99; found, N, 8.18

*dl-N-Pantoylglycine (Sodium Salt)*—The sodium salt of glycine was prepared by addition of glycine to sodium in liquid ammonia (12). Two methods used in the preparation of sodium pantothenate (13, 14) were employed in the synthesis of the sodium salt of *N*-pantoylglycine. Fusion of 1.34 gm. of the *dl*-lactone with 1.0 gm. of the sodium salt of glycine at a temperature of 120° for 3 hours gave a product of about 80 per cent purity. By refluxing for 3 hours a solution of 1.31 gm. of the *dl*-lactone and 0.98 gm. of the sodium salt of glycine in 7 cc. of isopropyl alcohol and then cooling, an equivalent yield of relatively pure product was obtained. On slow recrystallization from absolute ethanol, the compound formed hygroscopic colorless prisms which lost their crystalline character rather sharply at 94–96° and formed a glass which decreased in viscosity as the temperature was raised. This behavior is analogous to that of sodium pantothenate (14).

*Analysis*— $Na(C_8H_{14}O_5N)$ . Calculated, N, 6.17; found, N, 6.36

*Testing Methods*—Tests with yeast were performed as described by Snell *et al.* (15) for biotin assay. The medium was modified by addition of excess biotin (0.25  $\gamma$  per 5 cc.) and omission of  $\beta$ -alanine. With lactic acid bacteria, a previously described procedure was used (16). The medium (16) was modified by omission of pantothenic acid and addition of 1 mg. of asparagine, 10  $\gamma$  of pyridoxine hydrochloride, and 1  $\gamma$  of *p*-aminobenzoic acid per 10 cc. For *Escherichia coli* and *Staphylococcus aureus*, sodium acetate was omitted from this medium and the level of phosphates in-

creased 3-fold. Culture tubes were inoculated with washed cells from actively growing cultures (16 to 24 hours old) of the test organism grown in the basal medium, supplemented when necessary with pantothenic acid. Time and temperature of incubation are given with Tables I to IV.

*Results*—In a preliminary survey of various microorganisms, *Leuconostoc mesenteroides* P-60 proved to be suitably sensitive to the new antimetabolite, and was used to obtain most of the data. From Table I it is evident that pantothenyl alcohol has no growth-promoting action for this organ-

TABLE I

*Growth Inhibition by Pantothenyl Alcohol\* and Its Reversal by Pantothenic Acid*

Test organism, *Leuconostoc mesenteroides* P-60; incubated 22 hours at 30°.

Calcium pantothenate	Pantothenyl alcohol	Galvanometer reading†	Calcium pantothenate	Pantothenyl alcohol	Galvanometer reading†
$\gamma$ per 10 cc	$\gamma$ per 10 cc.		$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	
0.0	0.0	7.5	1.0	30	68
0.1	0.0	55	1.0	100	38
0.3	0.0	67	1.0	300	15
1.0	0.0	72	1.0	1,000	7
1000	0.0	74	0.0	10,000	7
0.1	3	55	30	10,000	13
0.1	10	32	100	10,000	40
0.1	30	15	300	10,000	70
0.3	10	67	1000	10,000	75
0.3	30	40	0.1	1,000†	59†
0.3	100	15	0.1	10,000†	63†

\* The condensation product prepared from the (–)-lactone was used in these experiments.

† A measure of culture turbidity, distilled water reads zero, an opaque object 100.

‡ Not pantothenyl alcohol, but the indicated amounts of both (–)-pantolactone and 3-propanolamine were added to each of these tubes. Condensation of the two to yield pantothenyl alcohol was effectively prevented by neutralizing the amine with hydrochloric acid before its addition. The slight stimulation of growth produced by this mixture at suboptimal levels of pantothenic acid is due to the pantolactone, which shows slight availability for growth at these high concentrations (cf. (4)).

ism, but rather inhibits growth. The level required to inhibit growth increases as the amount of calcium pantothenate is increased. Even large amounts (more than 10 mg per tube or 0.1 per cent) are non-toxic if sufficient pantothenic acid is present. The ratio of pantothenyl alcohol to calcium pantothenate at which inhibition is almost complete (the antibacterial index (17)) is about 300, and is constant over a wide range of concentrations. Mixtures of the (–)-lactone and 3-propanolamine have no such inhibitory effect.

In Table II, the inhibitory action of pantothenyl alcohol derived from condensation of 3-propanolamine with (-)-, (+)-, and *dl*-pantolactone is compared. The condensation product from the (-)-lactone is about twice as effective as that from the *dl*-lactone, and about 10 times as effective as that from the (+)-lactone. Thus growth inhibition by the condensation products shows the same configurational specificity as does growth promotion by pantothenic acid. The low activity of the product formed from the (+)-lactone may result from formation of some (-)-lactone by racemization in the alkaline reaction mixture.

TABLE II

*Comparative Activities of Pantothenyl Alcohol Derived from (-)-, (+)-, and dl-Lactones*

Test organism, *Leuconostoc mesenteroides*; incubated 21 hours at 30°. 1  $\gamma$  of calcium pantothenate was present in each culture tube (10 cc.).

Amount of product added  $\gamma$ per 10 cc.	Galvanometer reading* Pantothenyl alcohol†from		
	(-)-Lactone	(+)-Lactone	<i>dl</i> -Lactone
0	74	74	74
30	72	74	73
100	49	73	68
300	9.0	72	30
1000		43	4.0
3000		8.0	
Antibacterial index†	300 ca.	3000 ca.	>300 <1000

\* As in Table I; "blank" reading without added pantothenic acid, 6.0.

† The antibacterial index (17) is the ratio of the concentration of inhibitor ( $C_I$ ) to that of the essential metabolite (pantothenic acid,  $C_M$ ), at which complete inhibition of the test organism results.

The comparative potencies as growth inhibitors of products related in structure to pantothenyl alcohol are shown in Table III. The lower homologue of pantothenyl alcohol, N-pantoylethanolamine, has about one-fourth the activity of pantothenyl alcohol itself. In view of this fact, it is interesting that its oxidation product, N-pantoylglycine, which is structurally more similar to pantothenic acid, shows no inhibitory action. Inhibitory action of this nature evidently is not solely dependent upon structural similarity to the metabolite. This is further emphasized by the inhibitory properties of pantoylpropylamine, pantoylallylamine, and pantoylethylamine.

The comparative susceptibility of various organisms to growth inhibition by pantothenyl alcohol is shown in Table IV. Susceptibility among



organisms which require pantothenic acid varies greatly, from those which are very sensitive such as *Leuconostoc mesenteroides* and *Streptococcus lactis*, to those which are extremely resistant such as *Lactobacillus fermentum*, *Streptococcus faecalis* R, and *Saccharomyces cerevisiae*. None of the organisms tested which did not require added pantothenic acid for growth was inhibited by pantothenyl alcohol. The susceptibility of the same organisms to pantoyltaurine under the same conditions of growth was

TABLE III

*Growth Inhibition by Compounds Related to Pantothenyl Alcohol\**

Test organism, *Leuconostoc mesenteroides*; incubated 20 hours at 30°.

Amount of product added		Galvanometer readings†					
Inhibitor	Calcium pantothenate	dl-Pantothenyl alcohol	dl-N-Pantoyl-ethanolamine	dl-N-Pantoyl-n-propylamine	dl-N-Pantoyl-lallylamine	dl-N-Pantoyl-ethylamine	dl-N-Pantoyl-glycine
mg. per 10 cc.	γ per 10 cc.						
0.0	0.0	10	10	10	10	10	10
0.0	1.0	66	66	66	66	66	66
0.20	1.0	48	71	72	72		65
0.50	1.0	11	61	72	65	72	65
1.0	1.0	9.0	38	60	58	69	65
2.0	1.0		9.0	29	21	52	66
5.0	1.0			9.0	9.0	12	67
10	1.0					9.0	69
20	1.0						72
10	300	71	67	64	65	65	67
Antibacterial index‡		500	2000	5000	5000	10,000	>50,000§

\* The products tested were derived from the dl-lactone in all cases. None of them promoted growth in the absence of pantothenic acid. If it is assumed that only the condensation product derived from the (–)-lactone has inhibitory action (cf. Table II), products prepared from the (–)-lactone would be inhibitory at one-half the concentrations used above.

† As in Table I.

‡ As in Table II.

§ Separate experiments in which less pantothenic acid was present showed the antibacterial index of this product to be >500,000.

also determined. For the most sensitive organisms, the two antimetabolites have similar potencies as inhibitors; however, marked differences exist in the sensitivity of a given organism toward the two compounds. Thus *Leuconostoc mesenteroides*, which is extremely resistant to inhibition by pantoyltaurine, is the most sensitive organism to pantothenyl alcohol which was found. On the other hand, *Lactobacillus arabinosus* is much more sensitive to pantoyltaurine than it is to pantothenyl alcohol.

## DISCUSSION

Growth inhibition produced by both pantothenyl alcohol and pantoyltaurine (4) is specifically counteracted by pantothenic acid; whether or not inhibition of growth results is dependent upon the ratio of inhibitor to pantothenic acid. The relationship is competitive over a wide range. Both products appear to be completely non-toxic for organisms which

TABLE IV

Comparative Susceptibility of Various Organisms to Inhibition by Pantothenyl Alcohol and Pantoyltaurine

Organism*	Antibacterial index	
	Pantothenyl alcohol†	Pantoyltaurine‡
<i>Leuconostoc mesenteroides</i> P-60	300	>200,000‡
<i>Lactobacillus acidophilus</i> UT	5,000	5,000
" <i>arabinosus</i> 17-5	5,000	1,000
" <i>casei</i> . . .	10,000	10,000
" <i>fermentum</i>	100,000	>200,000‡
<i>Streptococcus faecalis</i> R	50,000	30,000
" <i>lactis</i> 125	1,000	
<i>Saccharomyces cerevisiae</i> FB	>30,000	10,000
<i>Staphylococcus aureus</i>	Not inhibited§	Not inhibited§
<i>Escherichia coli</i>	" " §	" " §

\* *L. mesenteroides*, *L. arabinosus*, *S. faecalis*, and *S. cerevisiae* were incubated at 30°; all other cultures at 37°. Incubation time was 21 hours in all cases. All of the organisms require pantothenic acid for growth except *S. aureus* and *E. coli*. Pantothenyl alcohol (or pantoyltaurine) did not replace pantothenic acid for any of these organisms. In all cases in which inhibition was produced, this was prevented by additional pantothenic acid.

† The products tested were prepared from (-)-pantolactone in each case and contained about 90 per cent of the condensation products. See reference (4).

‡ 20 mg. of pantoyltaurine produced no diminution in growth in the presence of 0.1  $\gamma$  of pantothenic acid. Higher levels were not tested.

§ These organisms were grown in a pantothenic acid-free medium; no inhibition of growth was produced by 30 mg. of pantothenyl alcohol or pantoyltaurine per 10 cc. of medium.

synthesize pantothenic acid. Because of these similarities, it might be assumed that their modes of action were similar. That the similarity is not complete is indicated by the data which show that organisms which are resistant to inhibition by pantoyltaurine may be sensitive to pantothenyl alcohol, and *vice versa*. No experimental data are available which explain this difference in action. It may result solely from differences in permeability of various microbial cells to the two substances. On the other hand,

the two compounds may interfere specifically with different reactions for which pantothenic acid is essential. In any case, the new antimetabolite might prove useful in investigating the mode of action of pantothenic acid.

Investigation has shown (18) that rats can be protected from as high as 100,000 lethal doses of injected hemolytic streptococci by pantoyltaurine. If, as has been stated by Pfaltz (10), rats can utilize pantothenyl alcohol as effectively as pantothenic acid, it seems unlikely that the former would have useful antibacterial action *in vivo*, since the host animal would produce from the antimetabolite the very metabolite which prevents its action. Much depends upon the site at which the various actions take place, however, and chemotherapeutic trial of the new substance should be interesting. In view of the general failure of the microorganisms tested to utilize the alcohol in place of the acid, this ability might not be generally distributed among animals. Further, oxidation of some of the compounds which show inhibitory action (e.g., N-pantoylethanolamine, etc.) to pantothenic acid is not possible.

Exchange of the carboxyl group of various metabolites for a sulfonic acid or sulfonamide group results, in many instances, in compounds which show inhibitory properties. From results cited above, it seems likely that a similar exchange of the carboxyl group of various essential metabolites for a hydroxymethyl group might result in a new class of inhibitory compounds.<sup>3</sup>

Recently, Barnett (7) has synthesized N-pantoyl- $\beta$ -aminoethylthiol and the corresponding disulfide. These compounds show inhibitory properties similar to those of pantoyltaurine. Although these compounds were synthesized because of their structural relationship to pantoyltaurine, they are just as closely related to compounds considered above. Thus N-pantoylaminoethylthiol is the thio- analogue of N-pantoylethanolamine. Test of the thio- analogue of pantothenyl alcohol should thus prove of interest.

Micro-Dumas and Van Slyke analyses were carried out by Gwyn White Shive.

#### SUMMARY

The preparation of pantothenyl alcohol, N-pantoylethanolamine, N-pantoylglycine, N-pantoyl-*n*-propylamine, N-pantoylallylamine, and N-pantoylethylamine is described. The latter four are new compounds and were obtained as crystalline solids. These compounds inhibit growth of some microorganisms which require pantothenic acid for growth. The

<sup>3</sup> Unpublished data show, for example, that *p*-aminobenzyl alcohol inhibits growth of some microorganisms; this inhibition is prevented by *p*-aminobenzoic acid.

inhibition produced is competitive in nature; progressively larger amounts of compound are required to inhibit growth as the amount of pantothenic acid present is increased. The sensitivity of the various microorganisms to the new antimetabolites varies widely. Of those tested, *Leuconostoc mesenteroides* was most sensitive. For this organism, the compounds vary as follows in their inhibitory powers: pantothenyl alcohol > pantoyl-ethanolamine > pantoylallylamine  $\geq$  pantoylpropylamine > pantoyl-ethylamine > pantoylglycine.

The susceptibility of various microorganisms to growth inhibition by pantothenyl alcohol and pantoyltaurine was compared. An organism may be very sensitive to one and very resistant to the other, although the mode of action of the two compounds appears very similar in other respects.

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# EFFECT OF THE INGESTION OF EXCESSIVE QUANTITIES OF CHOLINE ON THE AMOUNT IN TISSUES AND URINE\*

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It is well known that a deficiency of choline in the animal leads to the production of kidney lesions and to the accumulation of neutral fat in the liver. Patterson and McHenry (1) have reported that choline deficiency in young rats results in a decrease in the phospholipid content of the kidney. The same authors (2) later found that a deficiency of choline in young rats resulted in a 29 per cent decrease in the choline content of the kidneys and a 35 per cent decrease in the choline content of the liver. However, the amount of choline per 100 mg. of phospholipid was not appreciably changed, indicating that the amount of phospholipid formed is dependent on the amount of choline available.

Jacobi and coworkers (3, 4) on the other hand found no decrease in the choline content of the whole rat carcass after more than 8 weeks on a diet low in choline. It has been suggested that their results may be explained by the fact that the choline synthesis observed was due to the presence of other compounds in the diet capable of supplying methyl groups.

Fishman and Artom (5) have found that when weanling rats were placed on either a low or high fat diet the concentration of choline-containing liver lipids was markedly lower than that of rats on a stock diet. The addition of choline to the diets resulted in a pronounced increase in the levels of choline-containing liver lipids.

Since Luecke and Pearson (6, 7) have found that there are measurable amounts of free choline in plasma, liver, and kidney, it seemed desirable to study the effect of the ingestion of large amounts of choline on the free and total choline content of these tissues and the amount excreted in the urine. The total and free choline was determined by the microbiological procedure (6, 7).

\* The authors are indebted to Merck and Company, Inc., for the choline chloride and to the International Minerals and Chemical Corporation for the betaine hydrochloride used in these investigations.

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## EXPERIMENTAL

Sheep and dogs were used as the experimental animals, as relatively large amounts of blood are required for the determination of free choline. In the first series sheep weighing approximately 33 kilos were placed in metabolism cages and the 48 hour excretion of choline in the urine determined. Following this, 10 or 20 gm. of choline chloride in aqueous solution were administered by mouth. The urine was again collected for 48 hours and the amount of choline excreted determined. Samples of blood were drawn before the feeding of choline and at intervals of 1, 3, 5, 8, 24, and 48 hours thereafter. Both free choline and total choline were determined on the plasma. The animals were killed 48 hours after ingestion of the choline. The liver and kidney were analyzed for both free and total choline.

Three sheep were given 10 gm. of choline chloride and four were given 20 gm. Approximately 1 per cent of the choline ingested was excreted in the urine during the 48 hour period. The choline content of the feces was determined before and after feeding choline to a single animal. Only 1 per cent of the ingested choline could be accounted for in the feces. The total and free choline values of the plasma were virtually the same at the various intervals after feeding as they were before the ingestion of choline.

After the ingestion of choline the average value for the free choline content of the liver was 0.17 mg. per gm. and 0.28 mg. per gm. for kidney. These figures do not differ significantly from the average for five normal sheep of 0.20 mg. per gm. for liver and 0.29 mg. per gm. for kidney. The average values for the total choline of the sheep fed choline were 6.30 mg. per gm. for liver and 4.98 mg. per gm. for kidney. The corresponding values for liver and kidney from normal sheep were 6.25 and 5.00 mg. per gm. respectively. From these data it is evident that the ingestion of a massive dose of choline does not significantly increase either the total or free choline content of the liver or kidney.

Since only a small amount of a single dose of choline could be accounted for, we decided to feed 40 gm. daily for a period of 6 days. The choline and total nitrogen excreted in the urine and the free and total choline in the plasma were determined.

Table I shows the results of feeding 40 gm. of choline chloride daily to two sheep over a 6 day period. The maximum quantity of choline recovered in the urine in any 24 hour period amounted to only 2.5 per cent of the quantity ingested. It is also clear that the feeding of large amounts of choline has no effect on the free or total choline content of plasma. Analysis of the liver and kidney revealed no increase in either the total or free choline when compared with the values for normal animals.

Choline chloride contains approximately 10 per cent nitrogen; hence the ingestion of 40 gm. of choline chloride would increase the nitrogen intake

by about 4 gm. The urinary excretion of nitrogen increased over that of the basal period by 4 gm. per 24 hour period during the time choline was ingested, and in some instances the increase was even greater than could be accounted for on the basis of choline nitrogen fed. From this it is apparent that the animal stores no extra choline when excessive amounts are ingested. Since virtually all of the choline nitrogen is excreted in the urine and not more than 2.5 per cent as choline, it is evident that the animal rapidly metabolizes choline.

*Effect of Feeding Large Amounts of Choline to Dogs*—Two dogs, each weighing 7 kilos, were placed in metabolism cages and quantitative collec-

TABLE I

*Effect of Choline Feeding on Free and Total Choline Content of Plasma and Daily Urinary Excretion of Choline in Sheep*

40 gm. of choline chloride fed daily.

Sheep No.	Days fed choline	Choline in urine mg. per 24 hrs.	Choline in plasma	
			Free γ per ml.	Total γ per ml.
1	Initial	2	15.7	121
	1	306		
	2	352	14.2	132
	3	409		
	4	476	13.1	119
	5	425		
2	6	400	15.0	127
	Initial	2	11.1	141
	1	784		
	2	570	11.2	140
	3	1056		
	4	783	11.5	142
	5	650		
	6	600	10.8	139

tions of urine made for the 3 day period before supplementing the diet with choline, and for the 5 day period during which choline was fed. The amount of choline fed per day was 5 gm. It was incorporated in the ration with no effect on palatability, provided that some ground meat was included. The free and total choline values on plasma were determined on two samples, one drawn before supplementing the ration with choline, the other at the end of the 5th day of choline feeding. The results (Table II) show that the feeding of 5 gm. of choline chloride per day did not increase the concentration of either free or total choline in the plasma.

The urinary excretion of choline in response to the feeding of 5 gm. daily



of choline chloride is also shown in Table II. It is evident that the amount of choline recovered in the urine of the dog is even smaller than had been the case with sheep. The highest recovery of choline on any single day approaches 0.5 per cent. However, the percentage recovery in both species is so small that any difference between them is without significance.

*Comparison of Chemical and Microbiological Methods for Determination of Choline in Urine*—The most widely used chemical method for the determination of choline is the reineckate procedure. Ammonium reineckate is by no means a specific precipitant for choline (8). Thus, if the ingested choline were being excreted in any of the various nitrogenous forms which form a reineckate, the chemical method would give higher values than the micro-

TABLE II

*Effect of Choline Feeding on Free and Total Choline Content of Plasma and Daily Urinary Excretion of Choline in the Dog*

5 gm. of choline chloride fed daily.

Dog No.	Days fed choline	Choline in urine <i>mg. per 24 hrs.</i>	Choline in plasma	
			Free <i>γ per ml.</i>	Total <i>γ per ml.</i>
1	Initial	1.5	11.2	151
	1	20.2		
	2	19.5		
	3	12.2		
	4	25.2		
	5	30.0	11.0	150
2	Initial	4.0	9.5	120
	1	22.0		
	2	65.2		
	3	61.6		
	4	52.7		
	5	50.2	10.1	124

biological, since the latter is unaffected by these compounds. The reineckate procedure as described by Engel (9) was used for this purpose, the ammonium reineckate being added directly to 50 ml. of the undiluted urine. The resulting values, however, were in very close agreement with those obtained by the microbiological procedure, indicating that the extra nitrogen in the urine as a result of choline feeding was probably not in the form of a quaternary ammonium base, a tertiary, secondary, or primary amine.

*Effect of Ingestion of Betaine*—The work of Stetten (10) and others indicates that the methyl groups of betaine can be utilized for the synthesis of choline. The feeding of betaine might conceivably be accompanied by an

increase in the excretion of choline in the urine. Accordingly one sheep was fed 20 gm. of betaine hydrochloride for 2 successive days and the amount of choline in the urine determined by both the chemical and microbiological methods. Values obtained by the two methods agreed very well. The feeding of betaine hydrochloride was not followed by any increase in choline excretion, nor was any betaine excreted, as evidenced by the fact that the reineckate method which measures betaine showed no increase over that of the microbiological method which is unaffected by betaine. Thus, neither betaine nor choline was excreted as a result of betaine feeding.

#### DISCUSSION

It is difficult to explain the failure of liver, kidney, and blood to show any increase in either free or total choline when large doses of choline are fed. Undoubtedly some regulatory mechanism is present which very rapidly converts ingested choline to another metabolite. In this connection it is interesting to note that Jacobi and Baumann (4) suggested that their failure to find a decrease in the choline content of fatty livers as compared to the normal liver may be due to an inhibition of the choline oxidase as a result of the increased fat content. The work of Handler and Bernheim (11) showed that the activity of choline oxidase was depressed in the fatty liver. Thus, it may be that a reverse mechanism is being set up in the present case, and that the feeding of large quantities of choline stimulates the activity of this enzyme, thereby preventing any increase in the choline content of the tissues or blood.

#### SUMMARY

The ingestion of 40 gm. of choline chloride daily for a period of 6 days did not increase either the free or total choline content of the liver, kidney, or plasma. The choline recovered in the urine on any single day during choline feeding ranged from 0.7 to 2.5 per cent of the choline ingested by sheep. The amount excreted in the urine of dogs ingesting 5 gm. daily was approximately 0.5 per cent of the amount ingested.

The ingestion of choline is accompanied by an increase in urinary nitrogen. The increment in urinary nitrogen is virtually equivalent to the choline nitrogen ingested.

Betaine hydrochloride administered *per os* is not excreted in urine as choline or betaine.

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# THE INHIBITION OF CARBONIC ANHYDRASE BY THIOPHENE-2-SULFONAMIDE AND SULFANILAMIDE

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Since the discovery by Mann and Keilin (1) that sulfonamides having a free sulfonamide group inhibit carbonic anhydrase, it has been assumed that the inhibition of the enzyme accounts for the disturbances in acid-base balance following sulfanilamide administration. Also sulfanilamide has been used to test hypotheses concerning the rôle of carbonic anhydrase in several organs with positive results in the kidney (2) and negative results in the gastric mucosa (3, 4) and the pancreas (5). Since carbonic anhydrase is present in tissues in concentrations far in excess of those required (6), it is doubtful whether sulfanilamide in the concentrations used is capable of providing effective inhibition. The following data establish the facts that sulfanilamide cannot be expected to inhibit carbonic anhydrase sufficiently to interfere with processes of which the enzyme is a part and that another sulfonamide (7), thiophene-2-sulfonamide,<sup>1</sup> might be expected to do so.

*Inhibition of Carbonic Anhydrase Solutions*—Carbonic anhydrase was determined by the method of Meldrum and Roughton (8) in which a mixture of 2 ml. of 0.2 M phosphate buffer, pH 6.9, and 2 ml. of 0.2 M NaHCO<sub>3</sub>, is violently shaken, the rate of carbon dioxide evolution being followed manometrically. The enzyme unit is the amount of enzyme which produces a rate of evolution twice that of the uncatalyzed reaction during the third eighth of the reaction. All observations were made at 0° unless otherwise stated.

Carbonic anhydrase and its sulfonamide inhibitors enter into the reaction  $E + I \rightleftharpoons EI$ . The reaction is reversible, and equilibrium is reached almost instantaneously. The mass action expression for the reaction is

$$\frac{(E)(I - EI)}{(EI)} = K \quad (1)$$

The terms  $(E)$  and  $(EI)$  can be measured in terms of enzyme activity. The term  $(I - EI)$  is equal to  $(I)$ , for  $(I)$  is  $10^{-3}$  M or more, while  $(EI)$  is  $10^{-10}$  M or less. Therefore the constant is independent of the enzyme unit.

<sup>1</sup> I am deeply indebted to the American Cyanamid Company and to Dr. R. O. Roblin, Jr., for a generous supply of thiophene-2-sulfonamide.

The validity of equation (1) was proved by measuring the inhibition of carbonic anhydrase from laked washed red blood cells. The results are presented in Fig. 1. The curves drawn are those having a form derived from equation (1) and giving the best fit for the data. The value of  $K$  for thiophene-2-sulfonamide at  $0^\circ$  is  $1.6 \times 10^{-7}$ . It is the same at  $10^\circ$ , and the observed value can probably be safely used to calculate the inhibition at  $38^\circ$  with little error.

The value of  $K$  for sulfanilamide at  $0^\circ$  is  $1.0 \times 10^{-6}$ . A few observations at  $10^\circ$  gave a value of  $1.8 \times 10^{-6}$ . Extrapolation by means of the Arrhenius equation gave a value of  $7.4 \times 10^{-6}$  at  $38^\circ$ . Therefore sulfanilamide might

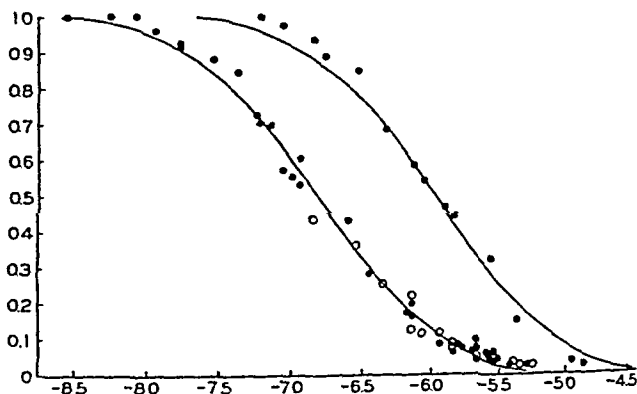


FIG. 1. Ordinates, fractional activity of carbonic anhydrase in the presence of inhibitors; abscissae, log inhibitor concentration in moles per liter. Left-hand curve, solid circles, thiophene-2-sulfonamide at  $0^\circ$ ; open circles, at  $10^\circ$ . Right-hand curve, solid circles, sulfanilamide at  $0^\circ$ .

be expected to be about 40 times less effective than thiophene-2-sulfonamide in inhibiting the enzyme in the body.

The values are independent of the purity or source of the enzyme, for identical values were obtained on very highly purified enzyme kindly supplied by Dr. D. A. Scott and Dr. F. J. W. Roughton and on enzyme from the kidney and gastric mucosa.

Equation (1) holds at very high enzyme concentrations. A reaction mixture was made containing 3675 units of enzyme and  $0.00199\text{ M}$  thiophene-2-sulfonamide. The expected activity was 0.30 unit, and the activity found was 0.78 unit. Similar experiments with sulfanilamide confirmed the prediction that the drug would be 6 times less effective than thiophene-2-sulfonamide at  $0^\circ$ .

A 4-fold variation in substrate concentration has no effect on the inhi-

bition. Two series of experiments were performed in which the reaction mixture was composed of 2 ml. of the phosphate buffer and 2 ml. of either 0.1 M or 0.4 M  $\text{NaHCO}_3$ . The results are presented in Fig. 2.

*Inhibition of Carbon Dioxide Uptake by Blood*—A comparison of the effectiveness of thiophene-2-sulfonamide and sulfanilamide in inhibiting carbonic anhydrase in cells was obtained by studying the rate of carbon dioxide uptake by blood. A reaction mixture was prepared by mixing 3 ml. of dog blood and 1 ml. of 0.9 per cent  $\text{NaCl}$  containing the desired amount of inhibitor. The mixture was violently shaken at  $0^\circ$  for 15 minutes while a steady current of air was blown through the reaction vessel. At the end of this treatment the blood was in equilibrium with air. Shaking and

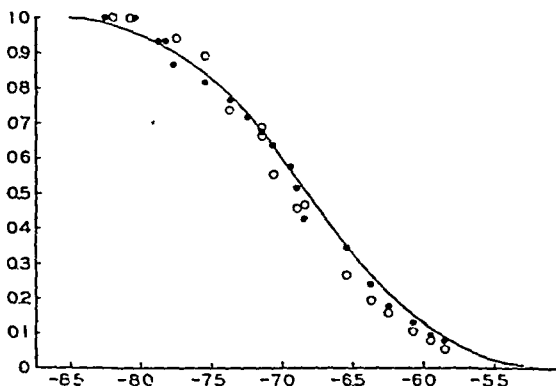


FIG. 2. Ordinates, fractional activity of carbonic anhydrase in the presence of thiophene-2-sulfonamide at  $0^\circ$ ; abscissae, log inhibitor concentration in moles per liter. Open circles, 2 ml. of 0.4 M  $\text{NaHCO}_3$  in the reaction mixture; solid circles, 2 ml. of 0.1 M  $\text{NaHCO}_3$  in the reaction mixture. The curve is identical with the left-hand curve in Fig. 1

aeration were stopped, and after the blood had drained from the sides of the vessel 2.2 ml. of carbon dioxide were admitted. The partial pressure of carbon dioxide was 17.7 mm. of Hg. Shaking was again begun, and the uptake of carbon dioxide was followed manometrically.

The results are presented in Table I. The concentrations of the inhibitors are given in mg. per cent for the sake of conformity with the rest of the literature on sulfonamide drugs. The rates of uptake of carbon dioxide are those observed in the second quarter of the reaction. The rates are corrected by the method of Roughton (9) for the limiting effect of diffusion through the gas-liquid interface. The second column under each drug gives the per cent inhibition of the catalyzed reaction calculated on the assumption that the slowest rate observed represents the uncatalyzed

alyzed reaction. The third column gives the per cent inhibition of the enzyme calculated from equation (1) and the appropriate constants.

These observations show that the inhibition of the catalyzed reaction is not proportional to the inhibition of the enzyme. In order to reduce the rate of the catalyzed reaction by 90 per cent more than 99.97 per cent of the enzyme must be inhibited. At 0° concentrations of thiophene-2-sulfonamide between 10 and 25 mg. per cent will effect this degree of inhibition, while the concentration of sulfanilamide must be over 50 mg. per cent. At body temperature much higher concentrations of sulfanilamide would be required.

TABLE I

*Rate of Uptake of Carbon Dioxide by Blood at 0° in Presence of Thiophene-2-sulfonamide and Sulfanilamide*

Inhibitor (I)	Thiophene-2-sulfonamide			Sulfanilamide		
	Rate of CO <sub>2</sub> uptake	Per cent inhibition of catalyzed reaction	Per cent inhibition of enzyme	Rate of CO <sub>2</sub> uptake	Per cent inhibition of catalyzed reaction	Per cent inhibition of enzyme
mg. per cent	c.mm. per sec.			c.mm. per sec.		
0	14.65	0	0	14.65	0	0
1	5.09	69.5	99.74	13.23	10.3	98.28
10	2.72	86.5	99.97	7.17	54.4	99.83
25	0.88	100.0	99.99	5.60	65.8	99.93
50	0.88	100.0	100.00	3.29	82.5	99.97
125				2.04	91.6	99.98
250				1.46	95.7	99.99

## SUMMARY

The inhibition of carbonic anhydrase by thiophene-2-sulfonamide and sulfanilamide can be described by the equation  $E + I \rightleftharpoons EI$ . The constant for the mass action expression of this reaction has been determined.

Thiophene-2-sulfonamide is 6 times more effective in inhibiting carbonic anhydrase at 0° than sulfanilamide and probably about 40 times more effective at 38°.

4-Fold variations in the substrate concentration have no effect on the inhibition.

In order to reduce the rate of the catalyzed uptake of carbon dioxide by blood at 0° by 90 per cent the carbonic anhydrase in the red cells must be inhibited by more than 99.97 per cent. The concentration of thiophene-2-sulfonamide required is between 10 and 25 mg. per cent, while the concentration of sulfanilamide is over 50 mg. per cent.

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# A SIMPLIFIED METHOD FOR THE DETERMINATION OF IRON IN MILK\*

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During studies on anemia in dogs, it was found necessary to determine the iron content of milk routinely. Since the present techniques for the determination of iron in milk generally include ashing, a new method was sought in which this step could be eliminated.

The method of Fowweather (1) was available, but wet ashing with sulfuric acid and hydrogen peroxide is still time-consuming. Barkan and Walker (2) found that digestion of blood plasma at 32° with 1.2 per cent hydrochloric acid gave reliable results. However, since this digestion fails to release firmly bound iron, it did not appear suitable for the determination of iron in milk. Tompsett (3) described a colorimetric method for the determination of plasma iron, using trichloroacetic acid to precipitate the protein material and thiolactic acid as the color reagent. This procedure requires an alkaline medium for the final development of color and cannot be applied to milk, since the addition of alkali to an acid extract of milk produces a heavy precipitate. However, within the course of his investigation, Tompsett (3) found that reduction of ferric iron with thiolactic acid or sodium hydrosulfite prevented the coprecipitation of the iron by proteins. In this connection, Borgen and Elvehjem (4) found that sodium pyrophosphate gave low iron values when added to a sample unless a reducing agent was also present.

Kitzes *et al.* (5) proposed a method in which the proteins are precipitated with heat and trichloroacetic acid, the buffered supernatant is reduced with thioglycolic acid, and the intensity of the color developed by the addition of  $\alpha, \alpha'$ -bipyridine is measured. As outlined, this procedure is not applicable to milk, since it does not liberate firmly bound iron. However, because it is rapid, accurate, and seemingly adaptable, attempts were made to modify the method for use on milk.

## Reagents—

1.  $\alpha, \alpha'$ -Bipyridine. 0.2 gm. of  $\alpha, \alpha'$ -bipyridine is dissolved in 5 ml. of c.p. glacial acetic acid and diluted with water to 100 ml.

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2. Ammonium hydroxide, approximately 6 N.
3. Buffer solution. Dissolve 27.2 ml. of c.p. glacial acetic acid and 33.4 gm. of c.p. sodium acetate in water and dilute to a volume of 250 ml.
4. Hydrochloric acid, 6 N and c.p., concentrated.
5. *p*-Nitrophenol, 0.1 per cent solution in water.
6. Redistilled water.
7. Standard iron solution, prepared by dissolving iron wire in dilute hydrochloric acid.
8. Thioglycolic acid, Eastman's practical grade.
9. Trichloroacetic acid, 25 per cent solution in water.

#### EXPERIMENTAL

The method of Kitzes *et al.* (5) was tried as such, but extremely low iron values were obtained (0.13 to 0.18 mg. per kilo of milk). Recovery of added iron was likewise poor and averaged only 45 per cent. These results were attributed to coprecipitation of the inorganic iron and failure to liberate the firmly bound iron. The work of Barkan and Walker (2) suggested that hydrochloric acid might overcome the difficulty. Therefore, new determinations were made with the procedure as outlined by Kitzes except for the addition of 1 ml. of 6 N hydrochloric acid to the digestion mixture. This set of determinations gave values of 0.44 to 0.63 mg. per kilo of milk. Subsequently varying amounts and concentrations of hydrochloric acid were tried. It was found that the addition of 1 ml. of concentrated hydrochloric acid gave the best results, and that higher concentrations of acid were not advantageous.

Because of the earlier work of Tompsett (3) and Borgen and Elvehjem (4) it was decided to add a reducing agent to the digestion mixture. Thioglycolic acid was chosen as the reducing agent, because Shorland and Wall (6) and Borgen and Elvehjem (4) had demonstrated its ability to liberate hematin iron. 5 drops of thioglycolic acid were added to the hydrochloric-trichloroacetic acid digestion mixture and the procedure followed as before. Values obtained in this analysis ranged from 0.522 to 0.564 mg. per kilo of milk and recoveries of added iron averaged 85 per cent.

Under these conditions a clear supernatant could not always be obtained. Therefore, the effect of various concentrations of trichloroacetic acid, length of heating, and speed of centrifugation was studied. It was found that adding 2 ml. of 25 per cent trichloroacetic acid to the hydrochloric acid digestion mixture and heating for 5 minutes in a water bath (90-95°) brought about the formation of a flocculent precipitate which could be removed by centrifuging at 3500 R.P.M. for 15 minutes. It was further found that all reagents can be added simultaneously to the digestion

mixture without altering the iron values. Recovery of added iron in this case was found to range from 95 to 106 per cent.

As another check on the reliability of the method, the residues of several samples were ashed, boiled in hydrochloric acid, and their iron content determined by the bipyridine method. Only negligible amounts of iron (possibly due to contamination) were found.

### *Proposed Procedure*

To 5 ml. of whole milk in a 15 ml. centrifuge tube, add 5 drops of thioglycolic acid, 2 ml. of trichloroacetic acid, and 1 ml. of concentrated hydrochloric acid. A blank with all reagents is also prepared.

TABLE I  
*Iron Values for Raw, Market, and Evaporated Milk*

5 ml sample of milk	Sample No.	Aliquot	Center point reading	Galvanometer reading	Iron in aliquot	Iron per kilo of milk
		ml.			$\gamma$	mg.
Raw.....	1	10	63.5	93.5	1.05	0.520
".....	2	10	63.2	93.8	1.00	0.500
".....	3	10	63.0	94.0	0.97	0.490
Market.....	1	10	64.0	94.0	0.97	0.490
".....	2	10	63.5	93.5	1.05	0.520
".....	3	5	63.8	96.1	0.57	0.570
Evaporated....	1	10	64.0	87.1	2.29	1.144
".....	2	10	63.0	86.2	2.47	1.234
".....	3	10	63.7	87.0	2.30	1.150

Stir well and place in a water bath heated to 90–95° for 5 minutes and cool in water.

Stir thoroughly to break up the precipitate, and centrifuge at 3500 R.P.M. for 15 minutes.

Decant the supernatant into a 25 ml. glass-stoppered volumetric flask. Wash the precipitate with a mixture of 2 ml. of redistilled water, 1 ml. of trichloroacetic acid, and 1 ml. of concentrated hydrochloric acid, and repeat the heating and centrifuging as above.

To the combined supernatants add 1 drop of *p*-nitrophenol and ammonia slowly until the solution becomes yellow. Make acid with 1 or 2 drops of hydrochloric acid or until the yellow color disappears.

Add 1 ml. of buffer solution and dilute with water to 25 ml. Mix.

Pipette an aliquot (5 or 10 ml.) containing 1 to 3  $\gamma$  of iron into an Evelyn tube and make to 10 ml. with water.

Reduce any ferric iron present with 2 drops of thioglycolic acid, and

determine the center point reading (100 per cent transmission on the galvanometer scale).

Add 1 ml. of 0.2 per cent  $\alpha, \alpha'$ -bipyridine reagent and mix. Read each tube in the colorimeter, using the respective center point reading obtained previously.

Calculate the amount of iron present by reference to a standard curve (5).

To test the validity and reproducibility of the proposed method, determinations were made on several samples of market milk, raw milk, and evaporated milk. A few typical data are given in Table I.

Johnston (7) recently made an extensive study of the iron content of market milks, using the thiocyanate method of Stugart (8), and found the values to range from 0.114 to 0.650 mg. per kilo of milk. Although the values obtained in this investigation are higher than those obtained by Johnston, they do fall within the range reported by other workers (9-11).

#### SUMMARY

A modification of the bipyridine method which requires no ashing is proposed for the determination of iron in milk. Iron values obtained by this procedure range from 0.490 to 0.570 mg. per kilo of raw or market milk.

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# NOTE CONCERNING THE REVERSIBLE INACTIVATION OF PROLAN

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(Received for publication, January 2, 1945)

The reversal by hydroquinone of the partial inactivation of chorionic gonadotropin at the temperature of boiling water has been studied by Bowman and ourselves. We (1) have been unable to find the reactivation reported by Bowman (2). The possibility, suggested by Fraenkel-Conrat (3), that the degree of inactivation might account for the difference in results must be considered, although we also failed to confirm the degree of inactivation at the temperature of the water bath reported by Bowman, and in this our results agreed with the data of three earlier reports. Moreover, we found that hydroquinone under the experimental conditions described causes partial inactivation of intact gonadotropin. Rimington and Rowlands (4) recently reported that the gonadotropin of pregnant mare serum, which was partially inactivated by heat, was not reactivated by hydroquinone. The large percentage of recovery (between 35 and 50 per cent) of active material after boiling half an hour as reported by Bowman could only be reconciled with the results of all other workers, who report less than 10 per cent recovery, on the assumption that the inactivation is not of the first order. This possibility was tested (Table I, Experiment I) by comparing the inactivation of chorionic gonadotropin in a concentration of 25 I.U.<sup>1</sup> per cc. with 12-fold this concentration. Both preparations were placed in a boiling water bath and plunged into a cold water bath as soon as the temperature reached 100°. It required 65 seconds to cover the temperature increment 70–100° and 17 seconds to cover the increment 100–60°. The results clearly show that concentration is without effect upon degree of inactivation and that only 5 per cent of the original biologic activity survives. The results of Experiments II and III show that to obtain a degree of inactivation comparable to that reported by Bowman a temperature of inactivation below 65° is required. Attempts were made to reactivate this reaction product, which had suffered a 60 per cent loss in potency, with hydroquinone. The experimental conditions, with the exception of the temperature of inactivation, were exactly the same as previously described (1). In each case hydroquinone produced a decrease rather than an in-

<sup>1</sup> This was the lowest concentration that could be used to assure an adequate concentration for the bioassay.

crease in potency. Finally, inactivation of chorionic gonadotropin in an atmosphere of nitrogen<sup>2</sup> was compared under otherwise identical conditions with inactivation in an atmosphere of air. The results of Experiment IV indicate that the oxygen of the air does not enter into the inactivation, as has been assumed (2).

## SUMMARY

1. A 12-fold increase in concentration of hormone was without influence upon the extremely rapid inactivation of chorionic gonadotropin at the

TABLE I  
*Treatment of Chorionic Gonadotropin (Prolan) with Hydroquinone after Heat Treatment*

Experiment No.	Treatment of prolan	Dose	Uterine* weight, mean and standard deviation of mean	Recovery	Limit of per cent deviation 19 of 20 times
		mg.	mg.	per cent	
I	Control	0.01	29 ± 4		
	82 sec. at 70-100-60°, 25 i.u. per cc.	0.20	28 ± 5	5	±1.3
	Control	0.01	28 ± 5		
	82 sec. at 70-100-60°, 300 i.u. per cc.	0.20	31 ± 7	5.5	±1.4
II	Control	0.01	26 ± 3		
	5 min. at 64.2°	0.05	65 ± 9	42	±8
	5 " " 64.2°, hydroquinone 30 min.; dilution, immediate administration	0.05	39 ± 6	30	±8
III	Control	0.02	63 ± 7		
	10 min. at 63.1°	0.075	95 ± 9	38	±8
	10 " " 63.1°, hydroquinone 30 min.; dilution, immediate administration	0.075	65 ± 9	27	±6
IV	Control	0.02	47 ± 7		
	7 min. at 63.5°, air	0.04	56 ± 6	56	±12
	7 " " 63.5°, nitrogen	0.04	50 ± 8	53	±12

\* The bioassay was performed in exactly the same manner as described in the earlier publication (1).

temperature of the boiling water bath, indicating that the reaction was a denaturation, not an oxidation.

2. A product which was inactivated to the extent of 60 per cent of the original activity by heating in a bath at 63-64° was not reactivated by treatment with hydroquinone. The rate of inactivation at 63-64° was not decreased when performed in an atmosphere of nitrogen.

<sup>2</sup> Equilibration was performed in a closed system fitted with a Bunsen valve. The control tube and tube containing nitrogen were placed in a water bath to attain the same temperature before being heated in the thermostat at 63.5°.

3. The possibility that the degree of inactivation is a factor in the reported reversal by hydroquinone on partially heat-inactivated chorionic gonadotropin was not substantiated.

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# ON THE COLORIMETRIC DETERMINATION OF CREATININE BY THE JAFFE REACTION\*

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Creatinine has been determined colorimetrically by the Jaffe reaction (1) for many years. Yet there are no published data which indicate why the particular concentrations of reagents are used. Folin (2) in the first paper which describes the determination of creatinine in urine by this reaction simply states: "Als Resultat vielfacher Untersuchungen bezüglich der Reaktion des Kreatinins mit alkalischer Pikrinsäurelösung hat sich folgendes ergeben . . ." and Shaffer (3) similarly states, "As a result of several hundreds of determinations under different conditions we concluded that the optimum conditions are . . ." No further details are given in either instance. Nor does subsequent work indicate what trend might be expected in the color developed if the concentration of picric acid or alkali, the volume, etc., are altered. The following experiments were undertaken, therefore, to obtain such information with particular reference to the determination of creatinine in very dilute solution.

## EXPERIMENTAL

The color densities were measured throughout with a Klett-Summerson photoelectric colorimeter (4). Filter 54 obtained with the instrument was used. By actual measurement in a Beckman spectrophotometer (5) this filter exhibits a maximum absorption at about 525  $m\mu$ . This is about the best wave-length at which to measure this color since picric acid absorbs strongly above 510  $m\mu$  and the color due to creatinine does not absorb much light above 540  $m\mu$ .

The concentrations of creatinine used were such that the readings obtained would fall within the most sensitive range of the photoelectric colorimeter. The volumes of the sample of the creatinine solution and the reagents were selected arbitrarily so that the determination could be carried out in a Klett-Summerson colorimeter tube or in Pyrex or other test-tubes which could be adapted to the instrument, and so that ordinary volumetric transfer pipettes may be used.

The standard creatinine solutions were prepared fresh daily by the

\* This study was aided by a grant from the John and Mary R. Markle Foundation.

proper dilution of a stock standard containing 0.5 mg. of creatinine per ml. of 0.1 M HCl.

The picric acid solutions were prepared by diluting the saturated stock solution. These dilutions were carried out at  $25^{\circ} \pm 1^{\circ}$ .

The sodium hydroxide was similarly prepared by diluting a 50 per cent solution by weight to either 10 or 20 per cent. These solutions were then diluted to the desired strength.

*Effect of Varying Concentrations of Picric Acid and Sodium Hydroxide upon Color Developed*—The first experiments were carried out at a constant

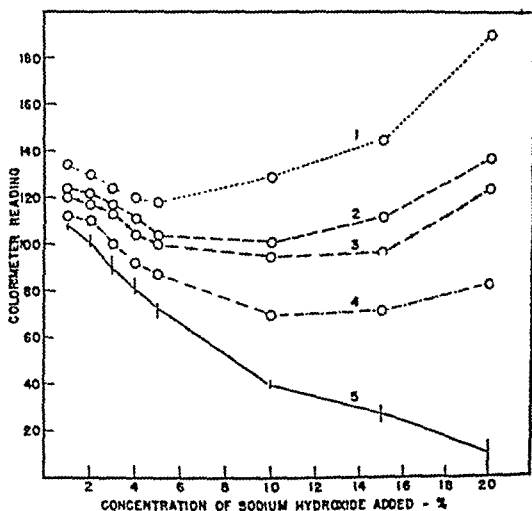


FIG. 1. Color developed with 20  $\gamma$  of creatinine as a function of the concentration of sodium hydroxide added at the following concentrations of 2 ml. of picric acid: Line 1, saturated picric acid; Lines 2, 3, and 4, one-half, one-third, and one-fifth saturated picric acid respectively; Line 5, average of values.

creatinine concentration as follows: 2 ml. of the picric acid solution and 1 ml. of the sodium hydroxide solution to be studied were added to 2 ml. of a solution containing 20  $\gamma$  of creatinine. The mixture was allowed to stand until the maximum color had developed. A blank determination in which water was used in place of the creatinine solution was prepared simultaneously in all cases. The color developed in both the standard solution and in the reagent blank was determined. The zero adjustment of the colorimeter was made with a tube containing distilled water.

The total color developed with different concentrations of picric acid at a constant creatinine concentration is plotted in Fig. 1 as a function of the concentration of the 1 ml. of sodium hydroxide added. The color

obtained (as read with the colorimeter adjusted to zero with distilled water) with 2 ml. of saturated picric acid is described by Line 1. Similarly, the color obtained with 2 ml. of one-half saturated picric acid is shown by Line 2; that with 2 ml. of one-third saturated picric acid by Line 3; and that with 2 ml. of one-fifth saturated picric acid by Line 4. These data seem to indicate that the color is a function of both the concentration of the picric acid and of the alkali. However, if the values obtained at the same concentration of creatinine and of added alkali but at different concentrations of picric acid are corrected for their own reagent blanks, the resulting values are essentially identical. The values so obtained in these

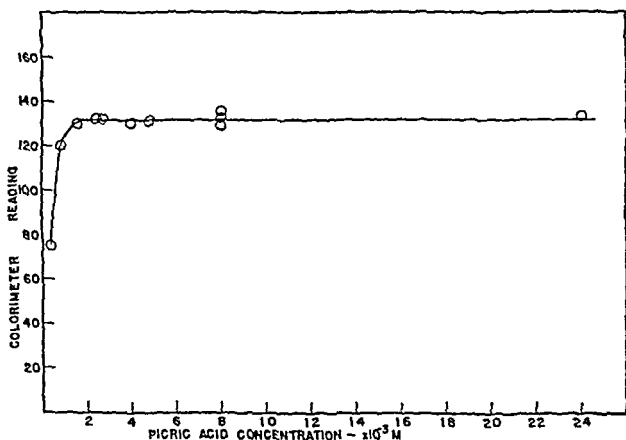


FIG. 2. Total color developed with 30  $\gamma$  of creatinine as a function of the picric acid concentration. Total volume 5 ml.; 1 ml. of 3 per cent sodium hydroxide used in all cases.

experiments have been averaged and are plotted as Line 5 in Fig. 1. The vertical lines indicate the range of values.

These data illustrate that the color obtained in the Jaffe reaction is independent of the concentration of picric acid, at least over a fairly wide range, and that it is dependent upon the concentration of the alkali employed. It is also apparent that as the concentration of the sodium hydroxide is increased the fraction of the total color attributable to creatinine is decreased, until at the higher concentrations of alkali nearly all the color is from the reagent blank.

Additional data are presented in Fig. 2 which show that the total color minus the reagent blank is independent, over a wide range, of the picric acid concentration at a constant alkali concentration. Since this graph includes data from experiments in which 3 ml. of creatinine solution and

1 ml. of picric acid were used as well as data from the experiments in which 2 ml. of picric acid were used, the picric acid concentrations are expressed in terms of moles of picric acid.

*Rate of Color Development*—The rate at which the color develops is inversely proportional to the concentration of both the picric acid and the sodium hydroxide; this is shown in Fig. 3.

*Proportionality of Color Developed to Concentration of Creatinine*—The relationship between the color developed and the concentration of creatinine was studied with several different concentrations of picric acid and alkali. As could be predicted from the previous data, the maximum color was obtained with 1 per cent alkali at all concentrations of creatinine.

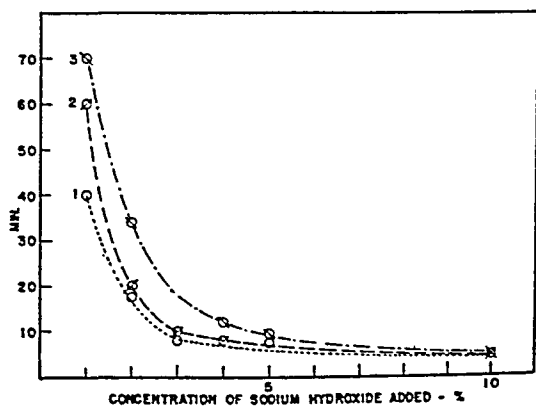


FIG. 3. The rate of color development as a function of the concentration of the sodium hydroxide added. 20  $\gamma$  of creatinine. Line 1, saturated picric acid added; Line 2, one-third saturated picric acid added; Line 3, one-fifth saturated picric acid added.

Progressively less color was also obtained at all concentrations of creatinine as more concentrated alkali was used. These data are shown in Fig. 4 where Lines 1, 2, 3, and 4 were drawn to act as visual guides to the data obtained at 10 to 50  $\gamma$  of creatinine when the alkali concentration (in the 1 ml. of sodium hydroxide added) was 1, 2, 3, and 4 per cent respectively. The concentration of picric acid used again seems to make little difference, although we have not studied this factor at all concentrations of alkali. However, the data indicated by Line 3 are the average results obtained in experiments in which 1 ml. of five different concentrations (saturated, one-half, one-third, one-fifth, and one-tenth saturated) of picric acid were used.

It is also apparent from these data that the color developed is not pro-

portional to the concentration of creatinine when the total volume is 5 ml. To substantiate this qualitative observation we have calculated the approximate molar extinction coefficients from the mean colorimeter readings obtained at creatinine concentrations of from 1 to 10 and at 20, 30, 40, and 50  $\gamma$  per 5 ml. with the creatinine method to be described below. These molar extinction coefficients are shown in Table I. The Lambert-Beer law seems to apply at creatinine concentrations below 10  $\gamma$  per 5 ml. (Fig. 4).

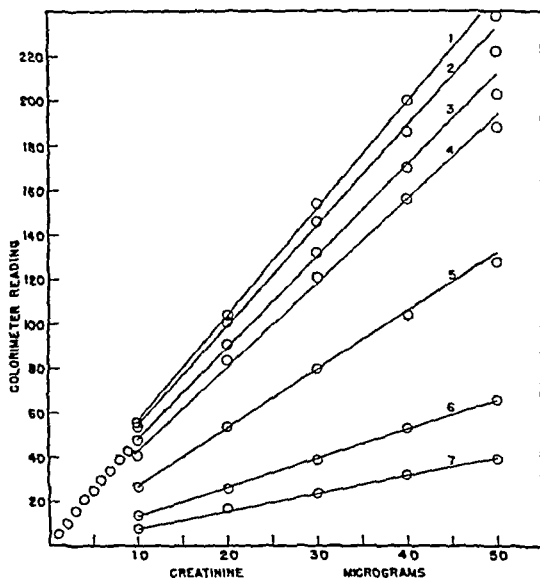


FIG. 4. Color developed as a function of the creatinine concentration. Lines 1, 2, 3, and 4 show color obtained with 1, 2, 3, and 4 per cent NaOH, respectively; Lines 5 and 6, result of diluting solutions from Line 3 to 2 and 5 times their original volumes with water; Line 7, the Folin-Wu method.

Above this concentration, however, the calculated extinction coefficients show a gradual decrease. The Lambert-Beer law, therefore, does not apply at these concentrations.

However, the proportionality between the color and the concentration can seemingly be improved by diluting the reaction mixture after the color has developed or by allowing the color to develop in a more dilute solution. For example, if the solution obtained in the creatinine method described below is diluted with an equal volume of water (*i.e.*, to a total volume of 10 ml.), the proportionality is improved somewhat. The color developed before dilution, in terms of colorimeter readings, is about the same as that

indicated by Line 3, Fig. 4. The colorimeter readings obtained after the solution is diluted with an equal volume of water are indicated by Line 5. If this reaction mixture is diluted still further to a total volume of 25 ml., the apparent proportionality is still better (Line 6) and the calculated molar extinction coefficients are approximately constant (Table I).

TABLE I  
*Molar Extinction Coefficients (Approximate)\**

Creatinine per volume analyzed	Creatinine per liter	Molar extinction coefficients, $\times 10^3$			
		Undiluted	Diluted		Folin method
			1:1	1:5	
$\gamma$	<i>micromoles</i>				
1	1.8	5.65			
2	3.5	5.65			
3	5.3	6.00			
4	7.1	5.95			
5	8.9	5.65			
6	10.6	5.65			
7	12.4	5.50			
8	14.2	5.50			
9	16.9	5.40			
10	17.7†	5.65	6.65	7.90	5.45
20	35.4	5.35	6.20	7.35	5.80
30	53.1	5.05	6.10	7.40	5.40
40	70.8	4.95	6.00	7.50	5.40
50	88.5	4.75	5.85	7.25	5.30

\* The colorimeter reading on the Klett-Summerson instrument multiplied by  $2 \times 10^{-3}$  gives a value which approximates  $D = (2 - \log G)$ . If one assumes that the cylindrical tube used in this instrument is approximately equivalent to a solution thickness of 1 cm.,  $D$  becomes approximately equal to the extinction,  $k$ , or more properly  $k'$ , where the prime indicates the inclusion of unknown correction factors. This  $k'$  divided by the concentration of creatinine in micromoles per liter is the approximate molar extinction coefficient described.

† This and the subsequent figures in this column must be divided by 6 for the Folin method.

Also, when the color is allowed to develop in a more dilute solution, as in the Folin-Wu method for blood (6), the proportionality between the color and the concentration is good (Line 7, Fig. 4) and the molar extinction coefficients are approximately constant (Table I). It is, however, immediately apparent that there is a decrease in both total color and in sensitivity when the Lambert-Beer law seems to apply.

*Application to Determination of Creatinine and Creatine in Diluted Urine and in Blood Filtrates*—We have selected for our own use one procedure from the many possibilities indicated above. The reasons which motivated

this particular selection are presented in the discussion. The values obtained by this procedure have been compared with the values obtained by the Folin methods for the determination of creatinine in urine (7) and in blood filtrates (6).<sup>1</sup>

The procedure employed by us follows: 3 ml. of the unknown solution (diluted urine or blood filtrate) containing up to 50  $\gamma$  of creatinine are used. To this solution is added 1 ml. of 0.04 *M* picric acid followed by 1 ml. of standardized 0.75 *N* (3 per cent) sodium hydroxide. The color is allowed to develop for 15 minutes. It is stable for at least another 30 minutes. The creatinine content of the solution is calculated from a standard curve obtained by averaging the results obtained in six different experiments at 1 to 10 and at 20, 30, 40, and 50  $\gamma$  of creatinine.

Added creatinine may be recovered quantitatively from the urine. Some typical results are shown in Table II.

TABLE II  
*Recovery of Added Creatinine from Urine*

Creatinine added	Creatinine found	Creatinine recovered
$\gamma$	$\gamma$	per cent
0	9.5	
5	14.4	98
10	19.8	103
20	29.7	101
30	40.0	102

In a series of thirty urines analyzed for creatinine by this procedure the average deviation from the values obtained by the Folin method (7) was -0.4 per cent, with a maximum range of  $\pm 10$  per cent. However, the majority of the determinations deviated less than  $\pm 5$  per cent from the value obtained with the Folin method.

Ten whole blood filtrates from patients who might be expected to have normal blood creatinine levels were analyzed by both methods. These bloods contained an average of 1.5 mg. per cent (range 1.4 to 1.7) of creatinine by the Folin method and 1.4 mg. per cent (range 1.2 to 1.5) by the procedure described above.

Creatine is 80 per cent converted to creatinine at these concentrations by heating the creatine-containing solution with picric acid in a boiling water bath for 45 minutes. This percentage conversion is of the same order of magnitude as that obtained in the Folin autoclave method. These observations confirm those of Albanese and Wangerin (8) who found that

<sup>1</sup> The color density in both these methods was measured with the photoelectric colorimeter. Standard curves were also used in both instances.



creatinine is not converted quantitatively to creatinine by the Folin autoclave method.

The percentage conversion is fairly constant and reaches a maximum value at about 80 per cent conversion when creatine is heated with picric acid in a boiling water bath, as described below. No creatinine is destroyed by this procedure. It seems, therefore, that for many purposes the procedure described below might serve as a method for the determination of creatine in dilute solution in that the procedure does not require an autoclave and requires no attention if a constant level water bath is available.

Creatine in urine is determined by the procedure described above for creatinine after heating 3 ml. of the creatine-containing solution with 1 ml. of 0.04 M picric acid in an unstoppered tube graduated at 5 ml. for 45 minutes in a vigorously boiling water bath.<sup>2</sup> After the solution has cooled to room temperature, 1 ml. of alkali is added. The volume is then adjusted to 5 ml. with water. To calculate creatine as creatinine the value obtained by difference in the usual manner is multiplied by 1.25. The values for total creatinine obtained by this method upon diluted urine were essentially the same as those obtained by the Folin autoclave method. If a quantitative conversion is required, the method described by Peters (9) might be applied, or the method described by Benedict (10) can be used before the urine is diluted. These procedures, however, have not been extensively studied by us. No attempt has been made to determine creatine in blood filtrates.

It appears, therefore, that the method described above for the determination of creatinine and creatine yields essentially the same values for creatinine and creatine in diluted urine and for creatinine in blood filtrates as do the Folin methods cited for the determination of these substances. This method has now been in use in our laboratory for the past several months for the routine determination of creatinine.

#### DISCUSSION

The ideal conditions for the determination of any substance by a colorimetric procedure are those which yield a color which is directly proportional to the concentration of the substance to be determined. The data presented above indicate that this ideal cannot be reached with this particular reaction regardless of the conditions chosen. However, one may approximate this ideal condition over the range of from 10 to 50  $\gamma$  of creatinine if procedures such as are described above (*i.e.* dilution) or the Folin blood method is used. But, it is doubtful whether dilution of

\*The water bath must be boiling vigorously.

the reaction mixture actually improves the proportionality. For when these reaction mixtures are diluted 5 times with water, the slope of the line describing the color as a function of the creatinine concentration is decreased to such an extent that the variations which occur probably fall within the resolving power of the instrument, since the measurements are made, perforce, at a constant solution thickness. So what appears to be an improvement in proportionality may be merely an artifact. Furthermore, when such procedures are followed, there is a great loss in sensitivity. For instance, when the Folin method is used, a difference of 40  $\gamma$  of creatinine results in a change of 31 scale divisions or 0.78 scale division per microgram. On the other hand, when the procedure described herein is used (assuming a linear relationship), a similar change in creatinine concentration results in a change in 161 scale divisions or approximately 4.0 scale divisions per microgram. It would appear, therefore, that if urines are diluted sufficiently so that the creatinine can be determined by the Folin blood method, as suggested by Peters (9), the low sensitivity of the method might lead to a relatively large error in the result. However, when blood filtrates are analyzed, one must choose between a method with a low sensitivity or a procedure which has greater sensitivity but which gives lower colorimeter readings.<sup>3</sup>

The assumption which has been made above that a linear relationship holds in the method described is obviously fallacious. However, it is of interest to calculate the actual percentage error which results if this assumption is made. For these calculations the mean values obtained at 10, 20, 30, 40, and 50  $\gamma$  of creatinine are compared with the values obtained from the best straight line which fits the data as calculated by the method of least squares. The values which are obtained from the straight line are then 6 per cent high at 10  $\gamma$ , 2 per cent low at 20  $\gamma$ , 1.5 per cent low at 30  $\gamma$ , 1.1 per cent low at 40  $\gamma$ , and 1.4 per cent high at 50  $\gamma$ . If the mean values are compared with the values obtained from the best straight line which fits the data between 10 and 40  $\gamma$ , the values obtained from the straight line are 2 per cent high at 10  $\gamma$ , 2 per cent low at 20  $\gamma$ , 0.75 per cent low at 30  $\gamma$ , and 0.58 per cent high at 40  $\gamma$ . It is possible, therefore, to use a formula<sup>4</sup> to calculate the creatinine concentration without introducing a very great error, even though a linear relationship is not found to exist.

<sup>3</sup> Similar results have been obtained when the Evelyn photoelectric colorimeter (11) has been used. To use this colorimeter all volumes are doubled. Filter 520 is used at Aperture 6.

<sup>4</sup> The mean values, the equations, and the formulas which may be derived from them are not presented because they are relative and not absolute values. It is not expected that identical numerical results can be obtained by others because of the variations which exist in filters.

However, one must be cognizant of the fact that errors are involved in such a procedure.

The particular method which we have outlined above was selected because it fits in best with our present analytical routines. The 0.04 M picric acid and the 0.75 N (3 per cent) alkali are used because the color develops sufficiently rapidly at these concentrations of picric acid and alkali and the blank color is still at a minimum. At the concentration of alkali at which the maximum color develops the rate of color development is too slow even with saturated picric acid for our purposes.

It is possible that at the concentration of alkali used more color may be produced from non-creatinine substances such as glucose than at lower concentrations of alkali. We have tested this point experimentally with respect to glucose. The color remains constant for 30 minutes after the maximum color has been attained with Folin-Wu blood filtrates and in urines to which 1 per cent glucose has been added. There may be an increase in color after 40 minutes. There is no difference in the creatinine values obtained in urine before and after the addition of glucose. The presence of glucose, therefore, does not seem to interfere with the determination.

Many questions concerning the mechanism of the Jaffe reaction are raised by the data. We feel, however, that there is no justification for a discussion of these points until more data are available.

#### SUMMARY

The effect of varying the concentration of picric acid and of alkali upon the color formed in the Jaffe reaction used for the determination of creatinine has been studied in solutions which contain 1 to 50  $\gamma$  of creatinine per 5 ml. The amount of colored creatinine compound formed as measured by the color developed is independent of the concentration of the picric acid added above a low limiting concentration of picric acid. The amount of colored creatinine compound formed is greatest at low concentrations of alkali and progressively decreases as the concentration of alkali is increased. The rate at which the color develops is inversely proportional to the concentration of both the picric acid and the sodium hydroxide.

The color formed is not directly proportional to the concentration of creatinine except at very low concentrations in the 5 ml. of solution employed. By diluting this solution the linearity seems to improve. This apparent improvement, however, is considered an artifact.

A procedure for the determination of creatinine in blood filtrates and in diluted urine is described which is based upon the data obtained.

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# THE COURSE OF THE ACID HYDROLYSIS OF GRAMICIDIN

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This paper deals with the hydrolysis of gramicidin by acid and the rate of appearance of various structures released by the rupture of linkages, including total amino nitrogen, periodate-labile amino nitrogen,  $\alpha$ -amino nitrogen, and alanine, and of the carboxyl group of leucine, and of valine and tryptophane utilizable by *Lactobacillus arabinosus*. The purpose of the study is to provide further information which may assist in determining the specific linkages of the constituent amino acids of the molecule. At the same time gramicidin offers a relatively simple test material for the study of various techniques for following the course of proteolytic hydrolysis.

## Methods

**Hydrolysis by Boiling Acid**—Gramicidin<sup>1</sup> was dissolved at a concentration of 10 mg. per ml. in glacial acetic acid (previously refluxed with potassium dichromate and redistilled). An equal volume of 6 N hydrochloric acid was added, and refluxing begun under a carbon dioxide atmosphere by lowering the flask into a bath of Wood's metal. Another volume of 6 N hydrochloric acid was added at a constant rate during the first 10 minutes of hydrolysis. At intervals during the subsequent hydrolysis, aliquots were removed without interruption of boiling or admission of air. These aliquots were at once evaporated to dryness *in vacuo* at 30° and dissolved in water for the analyses.

**Hydrolysis at 37°**—200 mg. of gramicidin were dissolved in 1 ml. of glacial acetic acid, and 1 ml. of 10 N hydrochloric acid was added. This solution was left for 10 days, at 37°, sealed in a glass ampul with a carbon dioxide atmosphere. The solution was then taken to dryness at low temperature and dissolved in water for analysis.

**"Complete" Hydrolysis**—The hydrolysis was carried out with the 1:2 mixture of acetic acid and 6 N hydrochloric acid in a sealed tube at 110° for from 46 to 48 hours to provide the standard for "complete" hydrolysis.

**$\alpha$ -Amino nitrogen** was determined by the procedure of Hamilton and Van Slyke (1) with the reaction with ninhydrin at pH 2.5. The solution used for absorbing the carbon dioxide contained hydrazine in addition to sodium chloride (2). The factors given by Van Slyke, Dillon, MacFadyen, and Hamilton (3) were used for calculating the results.

<sup>1</sup> Gramicidin has been isolated from tyrothricin supplied by Dr. Leo Wallerstein of the Wallerstein Company, Inc.

*Amino nitrogen* was determined by the manometric Van Slyke procedure (4). These determinations were also performed upon samples of hydrolysates which had been exposed to the action of periodate in dilute sodium hydroxide, the excess periodate being destroyed with glucose and the ammonia released being removed *in vacuo* prior to analysis.

*Alanine* was determined by measuring the acetaldehyde released by the action of ninhydrin according to the method of Alexander and Seligman.<sup>2</sup> In this method, the acetaldehyde is removed by aeration of the boiling solution, and measured by the color given with *p*-hydroxydiphenyl. Alexander has found that benzoylalanine and alanylglycine do not respond to this determination. Both the amino group and the carboxyl group are assumed to be required in the free state for acetaldehyde formation. The precision of these results was within 5 per cent of the values found.

*Release of Carboxyl Group of d(-)-Leucine*—Since only *l*(+)-leucine is appreciably active in stimulating the growth of *Lactobacillus arabinosus* under the conditions of the assays (5), it was necessary to racemize the samples prior to assay. This was done by treating aliquots of the hydrolysates with acetic anhydride for 48 hours according to the procedure of du Vigneaud and Meyer (6) for racemizing amino acids. After removal of the water, acetic acid, and acetic anhydride *in vacuo*, the products were hydrolyzed for 24 hours by 6 *N* hydrochloric acid at 115°. Aliquots were assayed for *l*(+)-leucine by use of *Lactobacillus arabinosus* according to the procedure of Hegsted (7).<sup>3</sup> The reproducibility of these assays was within 10 per cent. Available evidence indicates that in addition to free leucine, any peptide leucine having its carboxyl group free would be racemized by this procedure (9) and hence produce a growth response due to the *l*(+)-leucine formed.

*l*(+)-Valine and *l*(+)-tryptophane were also determined by the method of Hegsted (7)<sup>3</sup> by means of the growth response of *Lactobacillus arabinosus*. Each sample was assayed at four or five levels. The nephelometric readings at approximately 24 hours in most cases were used as the basis for measurement. Readings taken after other intervals and measurement of acid production gave concordant results. The rates of release of tryptophane and valine into forms available to the organism were determined in two or more separate hydrolyses.

*Destruction of Free Valine with Chloramine-T*—The microbiological assays may be assumed to measure both the free amino acid and amino acids contained in certain peptides. Although the composition of the pep-

<sup>2</sup> Alexander, B., and Seligman, A. M., unpublished work.

<sup>3</sup> Glycine, 0.5 mg. per tube, was added to the basal medium previously described. It may be noted that the values obtained for valine, leucine, and tryptophane after complete hydrolysis are in substantial agreement with values in the literature obtained by other methods (see Hotchkiss (8)).

tides that meet the nutritional requirements of *Lactobacillus arabinosus* for the various amino acids is unknown, the total of these is determinable by assay before and after free amino acids have been destroyed. Since both a free amino and a free carboxyl group appear to be required for the destruction of an amino acid by chloramine-T, most peptides should be stable to this reagent (3).

Chloramine-T was added in large excess to the above gramicidin hydrolysates which were buffered with phosphate at about pH 7.0. After 3 hours of incubation at 37°, residual chloramine-T was destroyed by adding an excess of glycine. The solutions were extracted three times with ether, taken to dryness *in vacuo*, and dissolved in water for microbiological assay. The fraction of the valine activity destroyed by chloramine-T is assumed to represent free *l*(+)-valine.

Samples of *l*(+)-valine and *l*(+)-leucine treated with chloramine-T under these conditions were destroyed quantitatively without producing any materials that interfered with the valine or leucine assays.

### Results

Table I summarizes the results of experiments in which hydrolysis was effected by boiling acid.

The determination of amino nitrogen and  $\alpha$ -amino nitrogen on the same hydrolysates provides standards for the over-all course of the hydrolysis, which may be used in three ways.

1. The measurement provided by the amino nitrogen determinations of the rate of splitting of all peptide links considered collectively furnishes a standard with which to compare the rates of splitting of peptide links of special categories as obtained by two other procedures: (a) the determination of racemizable leucine which is presumed to have measured the rate of splitting of links to which *d*(-)-leucine contributes its carboxyl group; (b) the determination of periodate-labile amino nitrogen which probably measured the cleavage of links to which the periodate-labile residue contributes an amino group. Nicolet and Shinn (10) found that substitution of a hydrogen atom of the amino group of serine by an acetyl group almost completely prevented attack by periodate. The rates at which these special peptide links are broken may be compared with the rate at which all of the links are split, as shown by amino nitrogen determinations.

2. The  $\alpha$ -amino nitrogen determinations measure the rate of release of free amino acids. These determinations furnish a standard with which the rates of release of any  $\alpha$ -amino acid can be compared.

3. Comparison of the values found for amino nitrogen and for  $\alpha$ -amino nitrogen for the same hydrolysates permits conclusions as to whether hydrolysis produces apparently random proportions of all the possible



amino acids, dipeptides, tripeptides, and other polypeptides, or whether the hydrolysis tends to produce either amino acids or oligopeptides selectively. Whereas amino nitrogen determinations measure the extent of cleavage of peptide links, the  $\alpha$ -amino nitrogen determination measures the occurrence of splitting of pairs of contiguous links so as to release free amino acids (amino acids originally at terminal positions being disregarded). The probability of two consecutive links being split is the square of the probability of the cleavage of any single link. Hence if  $n$  is the fractional

TABLE I

*Course of Hydrolysis of Gramicidin by Boiling Acid*

Values are per cent of the maximum reached by each analytical method in 46 to 48 hours. The per cent of gramicidin nitrogen in given forms at given times may be obtained by multiplying the percentages given in the table by the values at the foot of the respective columns.

Hydrolysate No.	Amino N		Periodate-labile amino N	$\alpha$ -Amino N	Free alanine	"Available" l(+)-valine			Free l(+)-valine*	"Available" l(+)-tryptophane		l(+)-Leucine†
	I	IX	IX	IX	IX	II	III	VI	X	VII	IX	IV
<i>min.</i>												
15		25.4	20	6.9	11.4		44	38	9	13	15	16
30	49	48	34	18.3	30.1	52	53		11	36	32	32
60		64	45	37.5	57	53	60		28	51	61	45
120	73	76	75	48.3	71	62	61		37		74	77
360	91	90	85	82.7	90	68	77		50		90	94
Fraction of gramicidin N represented by maximal release.....	0.803		0.20	0.726	0.106	0.077	0.081				0.38	0.094

\* Maximal values for free l(+)-valine, based upon reduction in available l(+)-valine produced by chloramine-T.

† l(+)-Leucine after racemization and further hydrolysis. These values are taken to measure the degree of release of the carboxyl group of d(-)-leucine.

extent to which the average peptide link is split (i.e., the fraction of maximal amino nitrogen attained), then  $n^2$  is the fraction of each amino acid which would be in the free state if splitting occurred at random points.

Considering the last of these three points first, we find the values of  $n^2$  are 0.065, 0.24, 0.42, 0.56, and 0.82 after 15, 30, 60, 120, and 360 minutes of hydrolysis at the boiling temperature. Comparison of these values with the rate of release of  $\alpha$ -amino acids indicates that somewhat less free amino acid was present at most points than would be predicted from the amino nitrogen values. A moderate tendency was shown for oligopeptides

to be formed in greater amounts and free amino acids in smaller amounts than the quantities predicted, assuming random cleavage. This disparity is greater if gramicidin contains any terminal amino acid residues. With the slow hydrolysis at 37° this tendency was stronger,  $\alpha$ -amino acids being released to 11.4 per cent of the maximum. The amino nitrogen determinations indicated that half of the peptide linkages were broken. If cleavage occurred at random points, one would expect amino acids to be freed to the extent of 25 per cent. Thus at 37° cleavage took place at weak points to a relatively greater extent than at the boiling temperature. One can conclude that these weak points tend not to be adjacent in gramicidin, or that cleavage of certain links inhibits the cleavage of adjacent links.

The rate at which *l*(+)-valine becomes available to *Lactobacillus arabinosus* deviated strikingly from the rate of release of the average amino acid. During the first 30 minutes 52 or 53 per cent of the maximum *l*(+)-valine became available; after this period the release became very sluggish. The yields of valylvaline isolated from gramicidin hydrolyzed for 2 and 6 hours under similar conditions (11) roughly approximated the unavailable valine at these two stages of the hydrolysis. Assays of concentrates of valylvaline from the hydrolysates have indicated that valine in this dipeptide is unavailable to the test organism.

*l*(+)-Tryptophane also became available to *Lactobacillus arabinosus* more rapidly than the average amino acid, although not so strikingly as in the case of *l*(+)-valine. That substantial portions of these amino acids available to *L. arabinosus* were still in the combined form can be seen by adding the available *l*(+)-valine, the available *l*(+)-tryptophane, and the free alanine. These sums during the early stages of the hydrolysis approach or exceed the  $\alpha$ -amino nitrogen found. Thus at 15 minutes the total of these three alone represents 9.8 per cent of the nitrogen of gramicidin, whereas only 9.5 per cent of the nitrogen was in the  $\alpha$ -amino form. The *l*(+)-leucine in leucylglycine and in glycylleucine has been shown to be available to *L. arabinosus* (5, 12). That large quantities of valine-containing peptides active for *L. arabinosus* were formed was shown by destroying the free amino acids in the various hydrolysates with an excess of chloramine-T. Subsequent assay showed that about three-fourths of the *l*(+)-valine activity found after 15 and 30 minutes of hydrolysis was due to compounds stable to chloramine-T. After 1 hour of hydrolysis 50 per cent, after 2 hours 40 per cent, and after 6 hours 31 per cent of the *l*(+)-valine activity was stable to chloramine-T.

There is one obstacle to the conclusion that the loss of *l*(+)-valine activity produced by chloramine-T was due entirely to destruction of free *l*(+)-valine. Destruction of tryptophane in tryptophane-containing pep-

tides probably results from the action of this reagent. Thus valine contained in peptides which also contain tryptophane may be made unavailable to *Lactobacillus arabinosus* because of modification of the indole group. Hence the results for chloramine-T-labile *l*(+)-valine activity are maximal values for the free *l*(+)-valine, and may include some peptide valine. This same consideration prevented the use of this reagent for the study of the state of tryptophane. Therefore, conclusions cannot be drawn as to whether the tryptophane utilizable by *Lactobacillus arabinosus* includes combined tryptophane. The values found for valine labile to chloramine-T demonstrate a striking retardation of the release of *l*(+)-valine during hydrolysis at the boiling temperature. The results also indicate the major degree to which combined *l*(+)-valine contributes to the *l*(+)-valine available to the test organism.

When the hydrolysis was performed for 10 days at 37°, *l*(+)-valine and *l*(+)-tryptophane activities for *Lactobacillus arabinosus* were respectively 58 per cent and 10 per cent of the maximum. Of this large fraction of valine which had become available, practically all was peptide valine; that is, stable to chloramine-T. This confirms Synge's finding<sup>4</sup> that very little free valine was formed under these conditions of hydrolysis.

Alanine was found present in the free state throughout the intervals studied, with hydrolysis with boiling acid, in proportions considerably in excess of the average amino acid, indicating a relative lability of the linkages in which this amino acid is involved. This lability was even more apparent with hydrolysis at 37°, alanine being released in 10 days to the extent of 22 per cent ( $\alpha$ -amino nitrogen, 11.4 per cent of the maximum). Thus both the relative stability of the linkages holding *l*(+)-valine and the lability of those holding alanine were accentuated by performing the hydrolysis at a lower temperature.

The rate at which *l*(+)-leucine appeared under the procedure employed, representing half of the *d*(-)-leucine in forms racemizable by acetic anhydride, was considerably slower during the 1st hour of hydrolysis than the rate of release of amino nitrogen, suggesting some lag in the splitting of those peptide links in which carboxyl groups of leucine are involved. The magnitude of the reduction of amino nitrogen produced by the action of periodate represented about 20 per cent of the nitrogen of gramicidin when hydrolysis was complete. This fraction is more than twice the difference between the amino nitrogen and the  $\alpha$ -amino nitrogen, and more than twice as great as the largest quantities of ammonia recovered from gramicidin hydrolysates by the action of periodate. A mixture of alanine, leucine, tryptophane, valine, and glycine simulating the proportions in gramicidin

<sup>4</sup> Synge, R. L. M., personal communication.

gave a small reduction (about 2 per cent) in amino nitrogen when treated with periodate under the same conditions.

Synge<sup>4</sup> observed that when the hydrolysis of gramicidin was first carried out for 10 days at 37° and then completed at 115°, the valine isolated had considerable optical activity, whereas with the usual hydrolysis by boiling acid, it had little or none. He suggested the possibility of a racemization involving both of the 2 contiguous valine molecules in gramicidin which could be avoided by the preliminary low temperature hydrolysis. If this were the case, one might be able to isolate an optically active form of valylvaline after the low temperature hydrolysis. At Dr. Synge's suggestion, an attempt was made to separate valylvaline from this hydrolysate. None could be obtained. Whether further hydrolysis would release this fragment has not been determined.

These results lend encouragement to the further attack upon the structure of gramicidin by the isolation of peptide fragments. The findings show that acid hydrolysis tends to produce oligopeptides selectively, and, further, the results indicate that certain linkages tend to be broken to a far greater extent than others. Both of these factors favor the possibility of discovering the sequence of the amino acids in the molecule by the study of fragments. The above data demonstrate the release of other unusually stable peptides of *l*(+)-valine in addition to valylvaline, which, in contrast to valylvaline, serve as a source of *l*(+)-valine for *Lactobacillus arabinosus*. Synge<sup>4</sup> reports that he has isolated from acid hydrolysates two other valine-containing peptides, *l*(+)-valylglycine and a tripeptide containing valine, leucine, and glycine. It is suggested that further interpretation of the foregoing data will be possible as more fragments arising from acid hydrolysis are recognized and their stability to acid and nutritional value to *Lactobacillus arabinosus* is determined.

#### SUMMARY

1. The hydrolysis of gramicidin by acetic acid-hydrochloric acid mixtures at the boiling temperature and at 37° has been studied by determinations of amino nitrogen,  $\alpha$ -amino nitrogen, free alanine, leucine racemizable by acetic anhydride, and of *l*(+)-valine and *l*(+)-tryptophane available to *Lactobacillus arabinosus*.

2. Comparison of the rates of release of amino nitrogen and  $\alpha$ -amino nitrogen indicates that hydrolysis tended to produce more oligopeptides and less free amino acid than would result from cleavage at random points, especially when the hydrolysis occurred at 37°.

3. *l*(+)-Valine appeared in forms available to *Lactobacillus arabinosus* much more rapidly than free amino acids in general were released. Much of this valine was in the form of peptides stable to chloramine-T, and, when

this stable fraction was deducted, the release of free *l*(+)-valine was seen to be unusually slow, especially at 37°

4. The release of tryptophane available to *Lactobacillus arabinosus* was somewhat more rapid than release of amino acids in general, except when hydrolysis occurred at 37°.

5. The linkages in which alanine is involved showed a relative lability under both sets of conditions of hydrolysis. Linkages attributed to the carboxyl group of leucine showed a relative stability during hydrolysis at the boiling temperature.

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# THE DETERMINATION OF URIC ACID IN HUMAN BLOOD

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Three factors have contributed to the uncertainty of the results of blood uric acid determinations by "direct" colorimetric procedures: (a) lack of proportionality between color intensity and uric acid content, (b) low recoveries of uric acid added directly to tungstic acid filtrates, and (c) lack of sufficient specificity.

Over 18 years ago the author indicated that better proportionality between color development and uric acid content could be attained if the reaction proceeded at room rather than at elevated temperatures (1). Subsequent modifications (2-4) have embodied this feature but true proportionality was, of course, not realized and the device of using correction factors or several comparison standards has been found necessary. More disturbing is the fact that quantitative recoveries of uric acid added directly to tungstic acid filtrates are not obtained with existing direct methods. Using the Blauch and Koch procedure (4), Bulger and Johns were able to recover only about half the added uric acid (5) and in our own hands neither it nor the Brown (1) or Folin (2) modification has yielded more than 82 per cent recoveries. Obviously substances present in tungstic acid blood filtrates inhibit color production under the conditions of the present direct methods and it is clear that, were it not for the additive effect of non-specific color formation, all uric acid values would be much lower than have been reported.

Attempts to overcome these discrepancies, which limit the usefulness of the method even for routine work, while at the same time retaining the simplicity of the direct procedure, have led to the development of conditions permitting recovery of about 95 per cent of reasonable amounts of uric acid added directly to tungstic acid filtrates of human blood, improved specificity, and a phosphotungstic acid reagent yielding relatively low blanks.

## *Solutions—*

*Standard uric acid.* The stock 0.1 per cent solution of Koch (6) was used. It keeps about a year. From it standards for use were prepared by simple dilution with water. In the cold, such water dilutions keep about 1 week; however, for intermittent use they are best prepared fresh. A 1:400 dilution gives a working standard equivalent to 2.5 mg. per 100 cc. of uric acid when equal volumes of a 1:10 tungstic acid filtrate and standard are used.

*Urea.* 50 gm. are dissolved in sufficient water to make 100 cc. The solution keeps well at room temperature.

*Sodium cyanide*, 12 per cent solution. In the cold it remains usable for about 2 weeks.<sup>1</sup> It should be brought to room temperature by immersion in warm water for a few minutes before use.

*Uric acid reagent.* 100 gm. of sodium tungstate and 20 gm. of anhydrous disodium hydrogen phosphate in a liter volumetric flask are dissolved with the aid of heat in about 150 cc. of water. Mix 25 cc. of sulfuric acid (sp. gr. 1.84) with about 75 cc. of water and pour the warm solution, slowly and with continual shaking, into the flask. Boil gently for 1 hour, using as a condenser a funnel holding a 200 cc. Erlenmeyer flask partly filled with ice water. Cool under running tap water and dilute to the mark with distilled water. The heated solution acquires a light greenish yellow tint which is considerably reduced on cooling and largely disappears as the result of dilution; boiling with bromine water to decolorize is unnecessary.

### Procedure

To 2 cc. of a 1:10 tungstic acid filtrate in a 10 cc. mixing cylinder or a Klett-Summerson test-tube graduated at 10 cc. are added 2 cc. each of the cyanide and urea solutions, followed by 1 cc. of the phosphotungstic acid reagent; mix after each addition by sidewise shaking. After standing 50 minutes at room temperature, dilute to the mark, mix, and compare in a colorimeter against 2 cc. of a uric acid standard similarly treated.

For visual colorimetry, if comparison is made against a standard equivalent to 2.5 mg. per cent it is simpler to set the unknown at 25; then one-tenth the reading of the standard is the uric acid content of the original blood.<sup>2</sup>

For photoelectric colorimetry both standard and unknown readings are corrected by subtracting the reading of a "blank" prepared from 2 cc. of water treated exactly as the unknown or standard. In this work a Klett-Summerson instrument was used with green Filter 54 and a distilled water "zero" setting. With a 2.5 mg. per cent standard  $2.5/(\text{reading of stand-})$

<sup>1</sup> Variation in color-enhancing properties of different lots of cyanide have been mentioned by various workers. Less attention seems to have been paid to the lack of keeping qualities of urea-cyanide mixtures. Such mixtures deteriorate rapidly even in the cold; blank values increase appreciably after about 36 hours. For this reason Blauch and Koch (4) use freshly prepared solutions; in the present method the urea and cyanide solutions are added separately.

<sup>2</sup> For uric acid values over 3.5 mg. per cent, or for instruments having large capacity cups, the unknown is set at 12.5; the standard reading is then, of course, multiplied by 0.2.

ard) (= factor<sup>3</sup>)  $\times$  reading of unknown = uric acid content of original blood.

#### EXPERIMENTAL

Preliminary tests indicated that improved recoveries of uric acid added to tungstic acid filtrates as well as better proportionality between uric acid content and color intensity could be obtained by decreasing the amount of uric acid and increasing the cyanide content (within certain limits) of the final reaction mixture. An added advantage that accrues under such conditions is that the time for maximum color development is thereby substantially reduced. But the already excessive blanks yielded by current phosphotungstic acid reagents are increased with increasing cyanide concentrations and a search was first made for a method of preparation that might produce a reagent free from this defect.

The principal change that has been made by various investigators in the original Folin-Denis reagent (7) has consisted, in the main, of successive reductions in its phosphoric acid content (4, 2, 8) but not much reduction in the blank value has thereby been realized except with the newer Folin reagent (8) which requires careful adjustment of its sodium tungstate content each time it is prepared. The phosphoric acid content of this reagent is less than half that of the original one of Folin and Denis but a consideration of Wu's work (9) indicates that a chromophorically active preparation should be possible with much less phosphoric acid.

When an aqueous solution of sodium tungstate and disodium hydrogen phosphate is heated with mineral acid, with the proportions suggested by Bailar (10) for the preparation of  $H_7P(W_2O_7)_6 \cdot XH_2O$ , a heavy white solid separates from the cooled reaction mixture. This does not react with uric acid; the filtrate, however, is active and it was found that an increase in the amount of phosphate produced less of the white solid with a filtrate having a greater chromophoric effect. By varying the phosphate and mineral acid content more than twenty different reagents were prepared and tested to determine the proportions that would yield maximum color with uric acid without a marked increase in blank values. The amounts that seemed to meet these criteria best contain just sufficient sulfuric acid to change all the tungstate and phosphate to free acids, with no precipitate of

<sup>3</sup> This factor is constant up to 6 mg. per cent (equivalent to 12  $\gamma$  in 2 cc. of a 1:10 filtrate) for any given set of reagents and the standard need be run only when new reagents are prepared. Since the blank value increases slowly but constantly during the useful life of the cyanide solution, it is best to run a blank with each day's set of unknowns. This also enables one to determine when a fresh cyanide solution should be prepared. For either method of comparison, if the unknown value exceeds 6 mg. per cent, the determination should be repeated with a 1 cc. sample.



inert white solid appearing in the cooled reaction mixture. Increasing the phosphate content above 20 gm. increases the blank values with but little increase in color formation due to uric acid. It is interesting to note that while this amount of phosphate supplies less than one-fifth the phosphoric acid used in the original Folin-Denis reagent it is still in considerable excess over the theoretical requirements for the chromophorically active tungstic acid complex (9). However, smaller amounts of both phosphate and mineral acid produced reagents with less activity and in all subsequent work the reagent as outlined under "Solutions" was used.

Within certain limits, the amount of color produced when phosphotungstic acid complexes react with uric acid increases with increase in cyanide concentration. However, two antagonistic factors appear to operate: the enhancing effect of cyanide on color production is opposed by the destructive action of the alkali on the color formed as the amount of cyanide is increased, and a cyanide concentration can be reached above which there is a net decrease in color formation, the colors becoming unstable and fading to weak greenish shades in a relatively short time. But even with optimum amounts of cyanide, proportionality between color production and uric acid content is attained only when the latter is low; the amounts of uric acid normally present in the 5 cc. samples of 1:10 tungstic acid filtrates customarily taken for analysis are too large to attain this desired proportionality.

These interrelationships were demonstrated as follows: 5 cc. portions of ten aqueous dilutions of the uric acid stock standard containing from 1 to 10  $\gamma$  per cc. were each treated with 2 cc. of 50 per cent urea, 2 cc. of cyanide solution, and 1 or 2 cc. of the phosphotungstic acid reagent. Without further dilution readings were made at 10 minute intervals in the Klett-Summerson colorimeter until constant values were obtained. Cyanide solutions of 10, 12, 15, and 18 per cent were used with each series of uric acid dilutions and the same procedure was repeated with 4, 3, and 2 cc. portions of the uric acid dilutions. When colorimeter readings were plotted against uric acid concentrations, curves of constantly changing slope were obtained in all cases except for the runs with the 2 cc. portions of the uric acid dilutions.

In Fig. 1 are plotted the colorimeter readings (corrected for blanks) against uric acid concentrations of five representative runs for 2 cc. samples with different amounts of cyanide and reagent. In these runs, readings were uniformly made after diluting to the indicated volumes 1 hour after the reagent had been added, since it had been found previously that the 1 hour waiting period was sufficient for maximum color formation if at least 2 cc. of 10 per cent cyanide were used, although less time is needed for maximum color production with the higher cyanide concentrations. Only

these five curves are shown, since they completely illustrate the various interrelationships; the curves obtained from runs with larger amounts of uric acid merely exaggerate the results. Fig. 1 shows that (a) a straight line passing through the origin is obtained for values of uric acid up to about 12  $\gamma$  when 2 cc. of 12 per cent cyanide and 1 cc. of reagent are used; (b) for cyanide concentrations above and below this amount somewhat less color is obtained and proportionality is not quite as good with the antagonistic

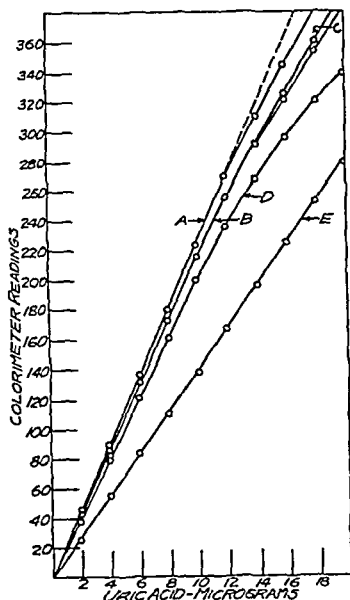


FIG. 1. Color development in relation to uric acid content after 60 minutes. Effect of variation in cyanide and reagent content. Curve A, 2 cc. of 12 per cent cyanide; Curve B, 15 per cent cyanide; Curve C, 10 per cent cyanide; Curve D, 18 per cent cyanide, each with 1 cc. of reagent and a final volume of 10 cc. Curve E, 2 cc. of 15 per cent cyanide plus 2 cc. of reagent in a final volume of 20 cc. The broken line indicates the extent and point of departure from the curve of constant slope of Curve A.

effect of excess alkali on color formation shown by the lower color values obtained with 18 per cent cyanide concentrations; (c) a straight line for all values up to at least 20  $\gamma$  of uric acid was obtained when 2 cc. of reagent were used.

Although proportionality of color intensity to uric acid content was best when 2 cc. of reagent were used, the results listed in Table I indicate that lower recoveries of uric acid added directly to tungstic acid filtrates were obtained than when a combination of 2 cc. of 12 per cent cyanide and 1 cc.

of reagent was used. The lower recoveries with both 10 and 15 per cent cyanide (with 1 cc. of reagent) seem to define the optimum cyanide concentration; apparently, increasing the cyanide content of the reaction mixture largely overcomes the color-inhibiting effect of tungstic acid filtrates of human blood, but 100 per cent recoveries are not obtained by increasing the cyanide content beyond certain limits, for then the destructive effect of the increased alkali on the colored complex leads to lower recoveries. Table I also points to the presence of larger amounts of color-inhibiting substances in rabbit blood filtrates, since uniformly poorer results were obtained with such filtrates.

TABLE I  
*Recovery of Uric Acid Added Directly to Tungstic Acid Filtrates*

Sample No.	Kind of blood	Uric acid		Per cent recovery, with 2 cc. of			
		In original filtrate	Added to filtrate*	12 per cent cyanide	15 per cent cyanide		10 per cent cyanide
		mg. per cent	mg. per cent				
1	Human	3.36	1.0	95.5	94.4	93.0†	91.3
2	"	3.28	1.0	95.0	93.2	92.5	90.8
3	"	2.02	2.0	93.2	92.4	90.6	91.0
4	"	2.45	2.0	94.8	93.0	93.0	88.2
5	"	2.30	3.0	93.8	92.1	92.0	87.6
6	"	1.90	3.0	94.5	92.8	92.0	92.0
7	Rabbit	0.45	3.0	91.5	91.7	92.8	88.0
8	"	0.53	3.0	91.6	92.2	93.1	87.5
9	"	0.58	4.0	91.2	91.0	90.6	86.3
10	"	0.65	4.0	90.8	91.4	91.4	86.6

\* Additions were made with uric acid solutions of such strength that they made up 10 per cent of the filtrates.

† The results in this column were obtained with 2 cc. of reagent; all others with 1 cc. of reagent.

With the optimum conditions observed above attempts were made to determine the effect of non-uric acid substances after the manner of Blauch and Koch (4). A solution was prepared containing, in mg. per cent, glucose 10, glutathione 4, ascorbic acid 0.2, and 0.2 each of tryptophane, tyrosine, cystine, and cysteine (calculated as amino nitrogen). 2 cc. portions were then treated as tungstic acid filtrates. The color intensity, calculated as uric acid, was equivalent to less than 0.3 mg. per cent. Ergothioneine was not available for inclusion in this solution but, since it has been shown (11) that tungstic acid precipitates variable but large amounts of this blood constituent, its effect can be expected to be minimum.

As a further test of specificity, uric acid determinations were made on a series of presumably normal human and rabbit bloods before and after

treatment with uricase (4). The results listed in Table II illustrate the small differences that were obtained; it would appear that, in the direct determination under the present conditions, non-uric acid color formation will increase the true uric acid content by less than 0.5 mg. per cent. The low values for rabbit bloods are also noteworthy, since direct methods hitherto used (1, 12) have resulted in much higher uric acid values.

The results for normal human blood listed in Table II have been divided into sex groups to illustrate the fact, apparent to us over many years of uric acid determinations, that lower values may be expected from blood of normal females. This is a consistent finding with any method and attention was called to it a few years ago by Bulger and Johns (5) and before that by Brøchner-Mortensen (13).

TABLE II  
*Uric Acid Content of Human and Rabbit Blood by New Procedure and after Uricase Treatment*

No. of samples	Kind of blood	Sex	Range of uric acid	
			New method	Removed by uricase
			<i>mg. per cent</i>	<i>mg. per cent</i>
12	Human	M.	2.22-3.45	2.05-3.10
17	"	F.	1.88-2.90	1.75-2.45
9	Rabbit		0.35-0.90	0.20-0.55

#### DISCUSSION

Since, in no instance, has 100 per cent of the uric acid added to tungstic acid filtrates been recovered, it is evident that the use of optimum amounts of cyanide in the reaction mixtures, while improving recoveries, does not completely overcome the color-inhibiting effect of substances in such filtrates. Nevertheless, it would appear that under the conditions described above about 95 per cent of the uric acid present in human blood filtrates will be determined with a minimum of interference from non-uric acid reactants.

No attempt has been made to determine the amount that can be recovered by this method when uric acid is added directly to whole blood or plasma, since it has been shown that uric acid is consistently lost by protein precipitation. The extent of such losses appears to depend upon whether whole blood or plasma is used (5) and perhaps on the manner of protein precipitation; such losses are, of course, independent of the method of determination.

It is noteworthy that the normal values for human blood obtained by this direct method correspond well to those found by the older Folin

isolation procedure (14) and considerable experience indicates that upper normal values do not exceed 3.5 mg. per cent.

#### SUMMARY

A new method of preparation of a phosphotungstic acid reagent suitable for use in uric acid determinations has been described. It incorporates less phosphorus and yields lower blanks.

An examination of the defects inherent in the direct determination of blood uric acid has led to the development of conditions, with the use of this reagent, which permit good recoveries of uric acid added directly to tungstic acid filtrates, strict proportionality between color intensity and uric acid content, and increased specificity.

Normal values for whole blood under these conditions do not exceed 3.5 mg. per cent.

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# THE PAPAIN DIGESTION OF NATIVE, DENATURED, AND "STABILIZED" HUMAN SERUM ALBUMIN

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Recent work in this laboratory has shown that the thermal stability of human serum albumin solutions, as measured nephelometrically and by the cloud point method (1), is increased by additions of salts of the lower fatty acids (1, 2). These results engendered an interest in other methods of measuring changes which may occur when the protein solutions are heated with or without added stabilizing agents.

Considerable work has been done (3-7) which demonstrates clearly that proteolytic enzymes hydrolyze denatured protein more rapidly than the corresponding native protein. Hence, it was thought that enzymatic studies might yield additional information about the state of the protein in heated solutions. Papain was chosen for the present study because it shows a marked difference in the rate of digestion of native and denatured protein (6), is stable to urea in high concentrations (6), and has a pH optimum which coincides with the pH optimum for thermal stability of human serum albumin.

The results of studies on the papain digestion of serum albumin denatured either by urea or heat, and with or without added stabilizers, are presented herein.

## EXPERIMENTAL

Clear solutions of activated papain, prepared by the method of Anson (8), were used. It was found that solutions of constant and reproducible activity could be readily prepared by this method from Merck's papain.

The substrate used was human serum albumin as prepared for the armed forces by the Plasma Fractionation Laboratory of Harvard University. Aqueous stock solutions containing 25 gm. per cent of albumin were prepared as previously described (1). Irrespective of the protein or urea concentration in the heated or urea-denatured solutions, all solutions were diluted so as to contain 2 gm. per cent of protein and 0.01 M phosphate buffer, pH 7.3, prior to digestion by papain. To 5.0 ml. of the 2 gm. per cent albumin solution in a test-tube was added 1.0 ml. of the enzyme solution, the contents were rapidly mixed, and the sample was incubated at

31°. The course of digestion was followed as described by Anson (8), with the Folin-Ciocalteu (9) phenol reagent for the colorimetric determination of the concentration of "apparent" tyrosine liberated. Readings were made with a Beckman spectrophotometer or a Klett-Summerson photoelectric colorimeter and converted to tyrosine equivalents by use of a standard tyrosine curve. All values were corrected for the blank or initial tyrosine value at zero time.

The sodium salts of the various fatty acids used in this work were prepared by neutralization of the corresponding acids.

#### RESULTS AND DISCUSSION

*Urea Denaturation of Serum Albumin*—In the first experiments the effect of denaturation by various concentrations of urea upon the rate of digestion of serum albumin was investigated. Solutions containing 2 gm. per cent of albumin were incubated for 24 hours with various concentrations of urea, and the rate of papain digestion was then determined as described above, with an undiluted 5.0 ml. aliquot. Preliminary experiments indicated that a digestion time of 5 minutes was satisfactory for comparison of the relative digestion rates. The results are presented in Fig. 1. This curve is similar to that obtained by Lineweaver and Hoover (6) with hemoglobin. It is apparent that for both human serum albumin and hemoglobin the maximum rate of proteolysis is produced by 6 M urea. The rate of digestion of albumin denatured by 6 M urea was assumed to be the rate characteristic of completely denatured albumin.

Variation of the protein concentration in the urea-protein solutions did not affect the proportion of protein denatured; samples incubated in 4 M urea with protein concentrations of 2 to 6 gm. per cent<sup>1</sup> showed no difference in the rate of digestion by papain after dilution to the same protein concentration of 2 per cent.

In all of the aforementioned experiments urea was present in high concentration during the papain digestion period. Control experiments demonstrated clearly that the urea itself had no adverse effect upon the action of papain. If the urea were removed by dialysis after the incubation period, the albumin underwent "regeneration" (4), and the rate of proteolysis was markedly decreased (Fig. 2). Similar results have been obtained in studies on tryptic hydrolysis of horse serum albumin and globulin (4).

*Heat Denaturation of Serum Albumin*—In the second group of experiments the papain digestion of heat-denatured human serum albumin was in-

<sup>1</sup> Relatively high concentrations of albumin in urea could not be used. For example, an 11 per cent solution of albumin in 4 M urea passed into a gel in less than 24 hours at room temperature.

vestigated. The results are given in Fig. 2. Although an increase in the rate of proteolysis could be obtained by heat treatment,<sup>2</sup> the maximum rate obtainable by heat denaturation was far less than that resulting from urea denaturation. In contrast, heat denaturation of hemoglobin has been reported to give a product which was attacked by papain about as readily

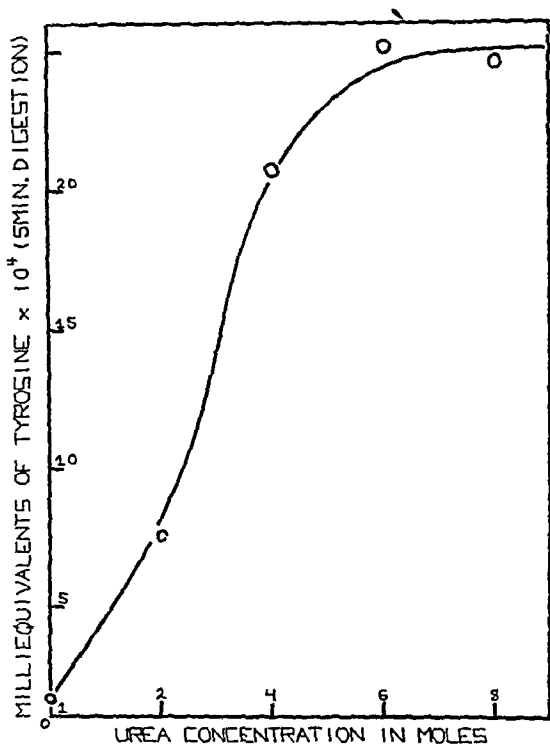


FIG. 1. The effect of urea concentration on the papain digestion of human serum albumin. The ordinate represents the milliequivalents of tyrosine in a 5.0 ml. aliquot of the filtrate from a mixture of 6.0 ml. of substrate and enzyme, and 10.0 ml. of 0.3 N trichloroacetic acid.

as urea-denatured hemoglobin (6). It is of interest that 25 gm. per cent solutions of albumin heated until they show a "cloud point" (1) do not show an increased rate of enzyme digestion. This indicates that in con-

<sup>2</sup> "Heat treatment," as used in the present paper, is defined as the heating of aqueous solutions of the protein under specified conditions of pH, salt content, protein concentration, temperature, and time.



centrated solutions only a small amount of albumin need undergo change for cloud formation to occur.

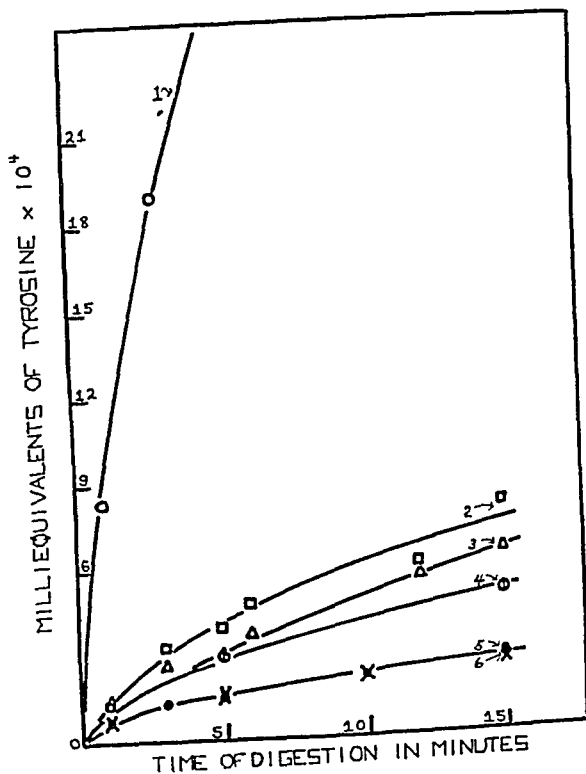


FIG. 2. Papain digestion of heat-coagulated human serum albumin (for comparison the digestion curves of native, urea-denatured, and regenerated serum albumin are included). Experiment 1 represents 2 gm. per cent albumin solution, pH 7.4, 0.01 M phosphate, stored in 6 M urea for 24 hours. Experiment 2, 25 gm. per cent albumin solution, pH 6.8, 0.15 M NaCl, heated at 100° for 3 minutes; coagulum then homogenized and diluted to 2 per cent protein for papain digestion. Experiment 3, 2 gm. per cent redispersed serum albumin, pH 7.0; heat-coagulated albumin (100°) from Experiment 2 redispersed by adding 0.1 N NaOH until dissolved, then adjusted with 0.1 N HCl to pH 7.0 (slightly turbid solution). Experiment 4, 1 gm. per cent regenerated serum albumin, pH 7.3; regenerated albumin prepared from urea-denatured product by dialyzing away the urea, heating the product at 40° for 15 minutes, and filtering. Experiment 5, 2 gm. per cent albumin, pH 7.3, 0.01 M phosphate. Experiment 6, 25 gm. per cent albumin solution, pH 7.0, 0.15 M NaCl, heated 2 minutes at 69° until heavily clouded; then diluted to 2 per cent protein for papain digestion.

*Effect of Sodium Caprylate on Results of Heat Treatment*—Sodium caprylate was chosen for these studies because the degree of stabilization

produced by fatty acids has been shown to increase with chain length up to caprylate, and caprylate in low concentrations has been found to be a satisfactory stabilizer for concentrated albumin solutions.<sup>3</sup>

4 gm. per cent solutions of serum albumin were heated for periods up to 27 minutes (2 hours in one case) at temperatures of 62°, 70°, and 78° with and without addition of 0.010 M sodium caprylate. Following heating and dilution to a protein concentration of 2 gm. per cent, the samples were digested with papain for 10 minutes, as in the preceding experiments. The purpose of the caprylate additions was to determine whether de-

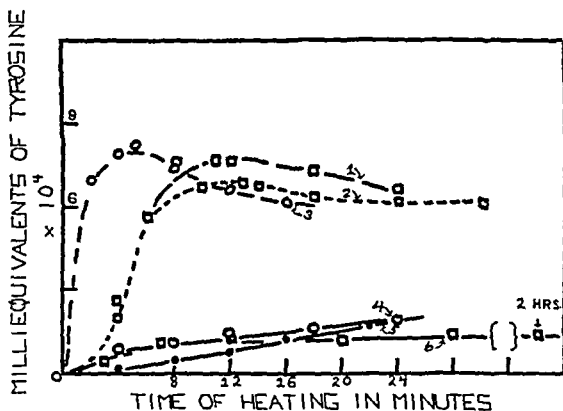


FIG. 3. Papain digestion of heat-treated human serum albumin (10 minute digestion period). Experiment 1, no caprylate, heated at 70°. Experiment 2, heated at 70°; caprylate (0.01 M) added to cooled solution after heating; sample became turbid after 7 minutes heating. Experiment 3, no caprylate, heated at 78°; sample became turbid after 2 minutes heating. Experiment 4, 0.01 M caprylate, heated at 78°; sample remained clear and limpid. Experiment 5, no caprylate, heated at 62°; sample remained clear and limpid. Experiment 6, 0.01 M caprylate, heated at 70°; sample remained clear and limpid. All samples contained 4 gm. per cent of albumin and 0.15 M NaCl, and were at pH 6.8. The values for tyrosine liberated are the difference between a control sample (not heat-treated) and the heat-treated solutions.

naturation, as measured by enzyme digestion rate, was decreased in the presence of caprylate. The results given in Fig. 3 show that solutions heated in the absence of caprylate were rapidly digested by papain as contrasted to the low digestion rate of the samples containing caprylate. The caprylate-stabilized protein displayed the same resistance to digestion by papain as albumin heated to a more moderate temperature in the absence of a stabilizer. The maxima in the uppermost curves roughly

<sup>3</sup> Boyer, P. D., Lum, F. G., Ballou, G. A., and Luck, J. M., unpublished data.

coincide with the times when the solutions in question became opaque and aggregation was noticeably apparent. The lower curves, not showing maxima, remained clear throughout the period of heat treatment. In the case of the former it may be inferred that coagulation, associated as it would be with decrease of surface area per unit mass, would at least tend to reduce the rate of digestion by papain. Experiments with human serum  $\gamma$ -globulin, which is more heat-labile than albumin, did not show a corresponding correlation between the appearance of the heated solutions and the ease of their hydrolysis by papain.

Experiments strictly comparable to the above with 25 gm. per cent solutions of human serum albumin were not feasible because such solutions gelled before showing any definite increase in susceptibility to enzyme hydrolysis. Solutions containing 25 gm. per cent albumin and 0.025 M caprylate which were heated for several minutes at 70°, as well as other samples which were maintained for upwards of 50 days at 57°, showed a rate of digestion by papain (after dilution to 2 gm. per cent) equal to that of unheated native serum albumin. Thus caprylate additions maintained these concentrated solutions in a native state as measured by susceptibility to enzyme digestion. In contrast, solutions without added caprylate readily formed opaque, solid gels under these conditions.

Addition of caprylate after heating the albumin reduced slightly but significantly the rate of digestion by papain (Experiment 6, Fig. 3). This effect is further discussed in a later section of this paper.

*Experiments with Other Stabilizers*—Solutions of 25 gm. per cent albumin, in the presence of other organic salts which have been shown to stabilize the albumin (1, 2), were similar in their behavior to solutions containing caprylate. At 70°, a temperature that requires only a few minutes to coagulate 25 gm. per cent solutions of human serum albumin without added stabilizing agents, 0.3 M concentrations of sodium butyrate, sodium phenyl acetate, sodium phenyl butyrate, and sodium mandelate, as well as 0.01 to 0.10 M concentrations of sodium caprylate, appeared to protect such solutions from the changes which otherwise occurred at that temperature. The papain digestion rates at 31° for such preparations, after the incubation at 70° for 25 minutes, were of the order of magnitude for native material. Storage of 25 gm. per cent serum albumin solutions containing 0.3 M sodium chloride or 0.15 M sodium chloride plus 0.15 M sodium butyrate at 50° for nearly 6 months, resulted in little effect on the rate of digestion by papain as subsequently determined.

*Effect of Certain Organic Salts on Urea Denaturation of Serum Albumin*—Upon further investigation of the urea denaturation of human serum albumin it was found that serum albumin, denatured in 6 M urea plus 0.02 M caprylate, showed a rate of digestion by papain but little higher than that

for native material. However, if the caprylate were added at the end of the 24 hour period used for the urea denaturation instead of simultaneously with the urea, the rate of enzyme digestion was very high, although still

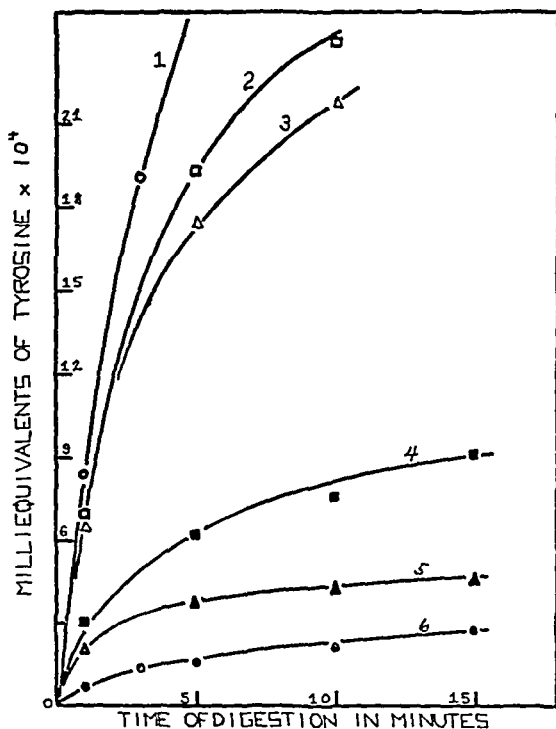


FIG. 4. Urea denaturation of human serum albumin; effect of stabilizers. Experiment 1, stored 24 hours in 6 M urea. Experiment 2, stored 24 hours in 6 M urea; subsequent adjustment to 0.020 M phenyl butyrate. Experiment 3, stored 24 hours in 6 M urea; subsequent adjustment to 0.020 M caprylate. Experiment 4, stored 24 hours in 6 M urea, 0.020 M sodium phenyl butyrate (urea and phenyl butyrate added simultaneously). Experiment 5, stored 24 hours in 6 M urea, 0.020 M sodium caprylate (urea and caprylate added simultaneously). Experiment 6, control; no urea treatment. All solutions contained 2 gm. per cent of human serum albumin and 0.01 M phosphate buffer; the pH was 7.4.

lower than that usually observed for 2 gm. per cent albumin denatured by 6 M urea. This experiment was repeated with the use of the other members of the series of organic anions studied and similar results were obtained for phenyl butyrate (see Fig. 4). Phenyl acetate, butyrate, and chloride

showed little effect, if any, on the urea denaturation of serum albumin. Preliminary tests on ovalbumin have indicated that the addition of caprylate had little effect on the course of its denaturation by 6 M urea.

The presence of caprylate has also been found to prevent the viscosity increase which otherwise occurs when albumin is denatured by urea.<sup>2</sup> Solutions containing 2 gm. per cent of albumin in 6 M urea had a relative viscosity indicative of an extended albumin molecule. In contrast, if 0.02 M caprylate were added before addition of the albumin, the resulting viscosity increase was considerably less and was of the magnitude which would be expected if the protein remained in a native state. Further experiments are planned on the study of the prevention of urea denaturation by lower fatty acids and related compounds.

The apparent protection of human serum albumin from heat or urea denaturation by low concentrations of caprylate is difficult to explain because the mechanism of denaturation is little understood. In the experiments with urea the relative number of molecules of urea, caprylate, and protein present was 21,000, 70, and 1, respectively. Thus it is apparent that the caprylate must exert its effect on the protein and not on the urea.

The increased susceptibility of denatured protein to proteolysis has been attributed to an extended configuration and to the exposure of more chemically reactive groups (4, 6), which renders the substrate more accessible to the enzyme. On the basis of the data presented in this paper it is probable that the addition of caprylate, or other compounds with a similar action, to albumin solutions prevents the liberation of active groups or extension of the molecule by heat or urea. Whether the mechanism of the action of caprylate in protecting serum albumin against heat denaturation and against urea denaturation is the same is not known, although these results do suggest a similarity in the action of heat and of urea. Studies on the nature of the association between caprylate and albumin are now in progress and may help elucidate the mechanism of the effect of caprylate. Incidentally, the evidence now presented requires us to modify our earlier hypothesis (1, 2) which suggested that the albumin molecule in its denatured, rather than its native, form associates with the stabilizer.

The small but significant reduction in the rate of papain digestion of urea-denatured or heat-denatured albumin brought about by the subsequent addition of caprylate or phenyl butyrate (Figs. 3, 4) may be due to steric or competitive inhibition effectuated through association of the stabilizer with the denatured molecule, or inhibition through association of the caprylate with the papain. The possibility that the caprylate may effect a partial return of the albumin to the native state also warrants consideration.

## SUMMARY

The rate of papain digestion, of human serum albumin denatured by urea or heat in the presence or absence of added stabilizing agents, has been studied. These experiments have yielded the following results:

1. Urea denaturation markedly increased the rate of papain digestion. Removal of the urea from the solution of urea-treated protein lowered the digestion rate nearly to the original level.

2. Heat treatment of albumin solutions, although resulting in an increased digestion rate, was far less effective than urea denaturation.

3. Additions of low concentrations of sodium caprylate or other similar stabilizing agents prevented the effects of heat treatment on susceptibility to digestion by papain.

4. Addition of caprylate simultaneously with urea apparently prevented the urea denaturation of the albumin as measured by enzyme susceptibility and viscosity increase.

This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

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# THE EXCRETION OF PYRIDOXINE, "PSEUDOPYRIDOXINE," AND 4-PYRIDOXIC ACID IN THE URINE AND SWEAT OF NORMAL INDIVIDUALS\*

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Snell, Guirard, and Williams (1) in 1942 showed that a metabolite of pyridoxine which they termed "pseudopyridoxine" was excreted in the urine. We have shown that this "pseudopyridoxine" occurs in sweat as well as in urine (2). "Pseudopyridoxine" was found to be inactive for the yeast, *Saccharomyces carlsbergensis*, used in the Atkin, Schultz, Williams, and Frey (3) assay method for pyridoxine, thus making it possible to determine pyridoxine in the presence of pseudopyridoxine. It has been shown (2, 4) that *Streptococcus lactis* R and *Lactobacillus casei* respond to pseudopyridoxine but not to pyridoxine, making possible a microbiological pseudopyridoxine assay, once a standard became available. In 1944 Harris, Heyl, and Folkers (5) synthesized "pyridoxal" and "pyridoxamine" from pyridoxine. Snell (6) showed that both of these compounds were active for the growth of *Streptococcus lactis* and that pyridoxal was active for the growth of *Lactobacillus casei*. Pyridoxal, therefore, appeared to be a better standard for pseudopyridoxine assay. This does not necessarily infer that pyridoxal is identical with pseudopyridoxine.

Huff and Perlzweig (7, 8) described, isolated, and characterized a third metabolite of pyridoxine, "4-pyridoxic acid" (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine). The existence of this compound in human urine had been indicated by Singal and Sydenstricker (9) in 1941. A fluorometric method for the determination of this metabolite has been described by Huff and Perlzweig (8).

In this paper we wish to report the excretion of pyridoxine, pseudopyridoxine (as pyridoxal), and 4-pyridoxic acid in the sweat and urine of young men maintained in a "hot moist" environment on a "normal" diet with and without added pyridoxine.

## EXPERIMENTAL

Four adult male subjects, age 21 to 28 years, were maintained for 8 hours per day under hot moist conditions for 5 days each week. The temperature

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.



of the experimental chamber was 37.8° and the relative humidity 70 per cent. The subjects were kept on a constant diet throughout the experiment. During Period II the diet was supplemented with 8 mg. of pyridoxine per day.

TABLE I  
*Average Daily Excretion of Pyridoxine and Its Metabolites*

Period No.	Subject	Pyridoxine	"Pseudopyridoxine" (pyridoxal)	4-Pyridoxic acid	Total
		mg.	mg.	mg.	mg.
I. Nodosage. Urine, per 24 hrs.	C	0.114	0.192	2.830	3.14
	D	0.187	0.375	3.022	3.58
	E	0.118	0.236	3.297	3.65
	F	0.131	0.181	3.367	3.68
Average.....		0.138	0.246	3.130	3.51
% of total.....		3.9	6.8	89.3	100
I. Skin excretion, per 8 hrs.	C	0.00	0.030	0.155	0.185
	D	0.00	0.030	0.193	0.222
	E	0.00	0.032	0.257	0.288
	F	0.00	0.035	0.189	0.224
Average.....		0.00	0.031	0.198	0.230
% of total.....		0	13.6	86.4	100
II. Dosage, 8 mg. pyridoxine per day. Urine, per 24 hrs.	C	0.354	0.460	7.540	8.35
	D	0.243	0.979	8.162	9.38
	E	0.409	0.716	6.220	7.34
	F	0.445	0.443	4.932	5.82
Average.....		0.363	0.650	6.713	7.72
% of total.....		4.6	8.4	87	100
II. Skin excretion, per 8 hrs.	C	0.0044	0.023	0.228	0.255
	D	0.0063	0.110	0.241	0.358
	E	0.0075	0.026	0.319	0.352
	F	0.0077	0.039	0.253	0.299
Average.....		0.0065	0.049	0.260	0.316
% of total.....		2.1	15.5	82.4	100

Complete 8 hour skin excretion collections and 24 hour urinary collections were made. The daily collections were composited for each 5 day experimental period. At the end of each experimental period, a 4 hour collection of undiluted sweat was made under the same environmental conditions.

The sweat and urine were analyzed for pyridoxine with a slight modification of the *Saccharomyces carlsbergensis* method of Atkin, Schultz, Williams,

and Frey (3). Pseudopyridoxine was determined with *Streptococcus lactis* R with the medium of Luckey, Briggs, and Elvehjem (10) for folic acid assay with the omission of pyridoxine and the addition of 80  $\gamma$  of folic acid concentrate<sup>1</sup> and 10 cc. of Salts B (11). Pyridoxal<sup>2</sup> was used as a standard for this determination. 4-Pyridoxic acid<sup>3</sup> was determined by the fluorometric lactone method of Huff and Perlzweig (8).

The average daily excretion in the urine and through the skin of each of these three metabolites for the two experimental periods is given in Table I.

From Table I it is apparent that 4-pyridoxic acid is quantitatively the most important metabolite excreted, amounting to over 80 per cent of the

TABLE II  
Concentration of Pyridoxine and Its Metabolites in Sweat

Post period No.	Subject	Pyridoxine	"Pseudopyridoxine" (pyridoxal)	4-Pyridoxic acid	Total
		$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.
I. No dosage	C	0.00055			
	D	0.00170	0.0825		
	E	0.00042	0.0043		
	F	0.00068	0.0105		
Average.....		0.00084	0.0324		
II. Dosage, 8 mg. pyridoxine per day	C	0.00049	0.0168	0.054	0.071
	D	0.0033	0.0145	0.048	0.066
	E	0.0016	0.0063	0.052	0.060
	F	0.0017	0.0090	0.055	0.066
Average.....		0.0018	0.0117	0.052	0.066
% of total.....		2.7	17.9	79.4	100

total pyridoxine and metabolite excretion. The daily excretion values for Period I seem high. However, these subjects had been receiving 2 mg. of pyridoxine daily in addition to their normal diet for several months prior to this experiment.

The sensitivity of the method for pyridoxine is such that the skin excretion for Period I may amount to 2 to 3 per cent of the total rather than zero.

In Table II are given the concentration data obtained on undiluted

<sup>1</sup> Dr. R. J. Williams, University of Texas, very kindly supplied the "folic acid" concentrate.

<sup>2</sup> Pyridoxal and pyridoxamine were generously supplied by Dr. Karl Folkers of Merck and Company, Inc., Rahway, New Jersey.

<sup>3</sup> 4-Pyridoxic acid was very kindly supplied by Dr. W. A. Perlzweig of Duke University.

sweat of the four subjects. These samples were collected during the 4 hour periods on the day following the conclusion of each experimental period.

The data in Table II indicate that the concentration ratio of the metabolites in sweat is about the same as in urine.

#### DISCUSSION

The average daily excretion value of pyridoxine and its metabolites under a continuous "hot moist" environment was 3.51 mg. in the urine, plus 0.69 ( $3 \times 0.23$  to convert from an 8 hour to a 24 hours basis)<sup>4</sup> through the skin, during Period I with no dosage. Thus 16.5 per cent of the pyridoxine plus metabolites was excreted through the skin. Similarly, for Period II, during which the diet was supplemented with 8 mg. of pyridoxine daily, the average total daily excretion was 8.67 mg., 11 per cent of this excretion occurring through the skin. While this 11 to 16.5 per cent excretion through the skin is a relatively small proportion of the total excretion, it cannot be definitely concluded that sweating conditions do not affect the pyridoxine requirement.

With the addition of 8 mg. of pyridoxine per day to the normal diet, the average increase in total excretion was 4.3 mg. or a 54 per cent recovery of the added pyridoxine. Of this increase, 5.5 per cent occurred as pyridoxine, 9.5 per cent as pseudopyridoxine, and 85 per cent as 4-pyridoxic acid, indicating that the added pyridoxine was metabolized in a similar manner to the pyridoxine present in the food.

Snell has shown (6) that pyridoxal and pyridoxine are approximately equally active for *Saccharomyces carlsbergensis*. Therefore, in order for pyridoxal and the pseudopyridoxine found in urine to be the same compound, the pyridoxine values would have to be higher in all cases than the pseudopyridoxine values obtained with *Streptococcus lactis*. In the work reported in this paper the pseudopyridoxine values were always considerably higher than the pyridoxine values. If, then, pyridoxal is not the same compound as pseudopyridoxine, the assumption that they are equally active for *Streptococcus lactis* may be possibly incorrect. Gunsalus, Bellamy, and Umbreit (12) have shown that a phosphorylated derivative of pyridoxal acts as the coenzyme of tyrosine decarboxylase. It may be that urinary pseudopyridoxine is such a conjugate of pyridoxal and is inactive for yeasts but retains its activity toward bacteria.

One of the questions arising from this study is the functional form of pyridoxine in the body. Scott *et al.* (13) have shown that the lactone of

<sup>4</sup> These would be maximal excretion values since they presuppose that there is no compensatory relationship between urinary excretion and skin excretion. Other work has indicated that this relationship frequently does exist.

"5-pyridoxic acid" ( $\alpha$ -pyracin) is active in preventing anemia in the chick. From the work reported here it would appear that some oxidation product of pyridoxine is possibly the active form in the body. Further work must be done to determine whether the final completely oxidized compound excreted in such large amounts is the functional form in the body or rather the final end-product of metabolism.

#### SUMMARY

1. A study was made of the excretion of pyridoxine, pseudopyridoxine (as pyridoxal), and 4-pyridoxic acid in the urine and sweat of four men subjected to a hot moist environment.

2. Over 85 per cent of the total pyridoxine and metabolites excreted in the urine was in the form of 4-pyridoxic acid, 4 to 4.5 per cent was pyridoxine, and 7 to 8 per cent was pseudopyridoxine.

3. The percentage distribution of these three compounds in sweat was similar to that in urine.

4. The total amount of pyridoxine and its metabolites present in sweat appears to be too small to have any significant influence on the pyridoxine requirement of persons subjected to an environment inducing profuse sweating throughout the day.

5. When 8 mg. of pyridoxine per day were given orally, an average of over 50 per cent of the pyridoxine was recovered in the sweat and urine as pyridoxine and its metabolites.

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# THE CHEMICAL COMPOSITION OF THE ADULT HUMAN BODY AND ITS BEARING ON THE BIOCHEMISTRY OF GROWTH\*

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The amounts of nutrients contained in the adult human body represent the integration of the day to day accretions from the time of conception to the termination of growth. These accretions are usually determined by balance experiments carried out at different periods of growth and are assumed to measure the net requirements of the respective nutrients with due consideration of the synthesis and transformation of organic nutrients in metabolism. Thus, the accretion of fat does not measure a fat requirement, but the accretion of protein and of the essential mineral elements may. Whether it does, or whether it does not, will depend upon the capacity of the body to store the nutrient in amounts considerably greater than current needs. The capacity of the body to store protein is strictly limited; so that nitrogen balances secured on a growing child after a reasonable period of adjustment to a liberal intake will measure reasonably well the amount needed for maximum growth in terms of net protein. The capacity of the body to store calcium is relatively enormous, far in excess of the needs of the soft tissues of the body and quite probably in excess of the need for a rigid and strong skeletal structure. Calcium balances on the child subsisting upon a liberal calcium intake may or may not measure the day to day need for net calcium, depending upon the degree of saturation of the skeleton in calcium salts, a condition that may vary with metabolic factors as well as with the food supply.

The extent to which metabolic balances of nitrogen, calcium, iron, etc., actually measure the day to day requirements in terms of net nutrients<sup>1</sup> can be judged by comparing their total integration throughout growth with the composition of the mature body with respect to them. However, information on the composition of the adult human body is strangely contradictory and incomplete.

The situation may be illustrated by the information on the content

\* The authors gratefully acknowledge the assistance to this investigation of funds donated by the Graduate School of the University of Illinois.

<sup>1</sup> By net protein, net calcium, etc., is meant the dietary supply minus all the losses to which the respective nutrient is subjected in the course of digestion, absorption, and assimilation as tissue constituents.

of calcium and phosphorus. The calcium and phosphorus contents of the adult human body have been variously estimated, often from undisclosed sources. Aron in 1908 (1) stated that adult man contains 4 per cent of ash, of which 40 per cent is  $\text{CaO}$ , the calcium content thus being 1.14 per cent. In 1919, Hackh (2) estimated the calcium and phosphorus contents of man at 1.90 and 0.95 per cent, respectively. 4 years later, Vernadsky (3) presented estimates of 1.4 per cent of calcium and 0.8 per cent of phosphorus, based on Volkmann's data published in 1874 and cited by Carl Voit in Hermann's "Handbuch der Physiologie," Leipzig, 1881. In a paper published in the following year, Vernadsky (4) cited some figures of Bertrand, reported in 1920; i.e., 1.38 per cent of calcium and 0.63 per cent of phosphorus. Gilbert and Posternak (5) state that the body of average adults contains about 1600 gm. of phosphoric acid, equivalent for a 70 kilo man to a phosphorus content of 1.0 per cent. From data secured from a rather complete collection of Ceylonese skeletons, Nicholls and Nimala-suriya (6) estimate the average calcium content of the adult male of Ceylon as 1.65 per cent and of the adult female, 1.52 per cent. Less direct calculations for the adult European led to values of 1.84 per cent calcium for the male and 1.34 per cent for the female. Leitch (7), on the basis of assumptions that seem none too probable, has calculated that the adult human body contains 36 gm. of calcium per kilo, or 3.6 per cent. From the fragmentary evidence available in the literature, Mitchell and Curzon (8) estimated the calcium content of man at 1.5 per cent, and at about the same time Shohl (9) presented an estimate of 1.66 per cent calcium and 0.96 per cent phosphorus.

Reflecting the confusion prevailing in the literature concerning the mineral content of the adult human body, Sherman, in his classical work, "Chemistry of food and nutrition," has estimated the calcium content at 2 per cent in the 1st and 2nd editions, 1.5 per cent in the 3rd, 4th, and 5th editions, and 2.2 per cent in the 6th and last edition published in 1941. The phosphorus content is set at 1.0 per cent up to the last edition, when it was raised to 1.2 per cent.

The scientific importance of information on the chemical composition of the adult human body and the wholly unsatisfactory character of that available prompted the authors to undertake the investigation reported in this paper. The plan involves the analysis of a number of human cadavers in the age range of 20 to 50 years and in satisfactory nutritive condition. Since such specimens are not readily available, publication of the results obtained on single specimens seems warranted.

#### *Methods and Materials*

The cadaver was obtained through the courtesy of Dr. Otto Kampmeier of the Department of Anatomy, College of Medicine, University of Illinois,

to whom grateful acknowledgment is made. It was that of a white man 35 years of age, 70.55 kilos in weight, and 183 cm. tall. Other measurements taken were the following: stem length (sitting height) 99.8 cm., shoulder (biacromial) width 35.3 cm., chest circumference at the level of the upper part of the xiphoid process 87.2 cm., and greatest distance between iliac crests 30.4 cm.

Death was due to an acute heart attack (decompensation or failure); postmortem examination performed by Dr. A. R. Cooper of the Department of Anatomy revealed passive congestion of both lungs, especially the lower lobes, and a moderately enlarged heart, showing evidence of chronic mitral valvulitis with mitral insufficiency or incompetency. "There were also a few small atheromatous white nodules in the lining of the first (ascending) part of the aorta, but the heart muscle showed no nodules. The twelfth rib on each side was only about 3 cm. in length, and the costal border was formed by the 9th ribs instead of the 10th." No other pathology or abnormality was noted.

Under the supervision of Dr. Cooper, the cadaver<sup>2</sup> was dissected into the various organs and tissues upon which separate weights and chemical analyses were desired. No attempt was made to remove all of the residual blood from the organs, although as much was removed as could be done by manual manipulation. The ulna, tibia, and ninth rib from the left side were analyzed separately from the other bones.

The samples were analyzed for moisture, nitrogen, ether extract, ash, phosphorus, and calcium by the official methods of the Association of Official Agricultural Chemists, except that in some of the soft tissues calcium was determined by a modification of the ceric sulfate method of Larson and Greenberg (10). The heats of combustion of all samples but the teeth were determined with the Parr adiabatic oxygen bomb calorimeter.

#### EXPERIMENTAL

Table I contains the values secured on the chemical composition of the organs and tissues analyzed, together with the relative weights of each with reference to the total body. The last row of figures relates to the composition of the entire body, which contained 67.85 per cent moisture, 12.51 per cent ether extract, 14.39 per cent protein ( $N \times 6.25$ ), 4.84 per cent ash, 1.596 per cent calcium, 0.771 per cent phosphorus, and a heat of combustion equivalent to 1.93 kilocalories per gm. Only 0.41 per cent is unaccounted for.

The skeleton, making up 14.84 per cent of the body weight, contains 30.1 per cent of the dry matter, 19.5 per cent of the fat, 18.6 per cent of the

<sup>2</sup> The body was preserved only by freezing until dissection was started about 6 weeks after death.



protein, and, with the teeth, 85.7 per cent of the ash, 99.0 per cent of the calcium, and 90.0 per cent of the phosphorus. The striated muscles constituted 31.56 per cent of the body weight, and contained 38.8 per cent of its water, 19.2 per cent of its dry matter, 8.1 per cent of its fat, 34.6 per cent

TABLE I  
*Chemical Composition of Adult Human Body*

Parts analyzed	Per cent of total body	Chemical composition						Heat of combustion
		Water	Ether extract	Crude protein (N $\times$ 6.25)	Ash	Calcium	Phosphorus	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>calories per gm</i>
Skin .	7.81	64.68	13.00	22.19	0.68	0.0205	0.060	2 292
Skeleton .	14.84	31.81	17.18	18.93	28.91	11.02	4.83	2 497
Teeth . . .	0.06	5.00*		23*	70.90	24.42	11.81	
Striated muscle	31.56	79.52	3.35	16.50	0.93	0.0099	0 116	1 239
Brain, spinal cord, and nerve trunks	2.52	73.33	12.68	12.06	1.37	0.0188	0.352	1 905
Liver	3.41	71.46	10.35	16.19	0.88	0.0102	0.148	2.196
Heart†	0.69	73.69	9.26	15.88	0.80	0.0078	0.113	1 824
Lungs‡	4.15	83.74	1.54	13.38	0.95	0.0116	0.114	0 985
Spleen..	0.19	78.69	1.19	17.81	1.13	0.0079	0.217	1 193
Kidneys	0.51	79.47	4.01	14.69	0.96	0.0130	0.174	1 326
Pancreas	0.16	73.08	13.08	12.69	0.93	0.0143	0.155	1.979
Alimentary tract	2.07	79.07	6.24	13.19	0.86	0.0125	0.115	1 339
Adipose tissue	13.63	50.09	42.44	7.06	0.51	0.0116	0.048	4 165
Remaining tissues								
Liquid .	3.79	93.33	0.17	5.68	0.94	0.0054	0.066	0.382
Solid..	13.63	70.40	12.39	16.06	1.01	0.0675	0.053	2.040
Contents of alimentary tract.	0.80							
Bile..	0.15							
Hair . .	0.03							
Total body, weighing 70.55 kilos.	100.00	67.85	12.51	14.39	4.84	1.596	0.771	1 930

\* Assumed.

† Somewhat enlarged.

‡ Somewhat congested.

of its protein, 5.8 per cent of its ash, only 0.2 per cent of its calcium, but 4.5 per cent of its phosphorus. Of the 1126 gm. of calcium\* in the entire body, all but 12 gm. are located in the bones and teeth, and probably one-half of this small residue is located in the ligaments and tendons (the solid "remaining tissues"). The ratio of calcium to phosphorus in the entire body was 2.07:1.

The total heat of combustion was 136,163 kilocalories.

The composition of the skeleton and of the three bones analyzed separately, computed on the bases of fresh weight, dry weight, dry and fat-free weight, and ash, is summarized in Table II. These data will be used in assessing the nutritional status of the cadaver specimen with reference to calcium.

*Normality of Specimen*—The significance of the data presented in the preceding section to any study of the biochemistry of growth will depend upon the normality of the material analyzed, particularly with reference

TABLE II  
*Chemical Composition (Per Cent) of Bones on Different Bases*

Constituent	Fresh basis	Dry basis	Dry, fat-free basis	Ash	Fresh basis	Dry basis	Dry, fat free basis	Ash
Left tibia					Left ulna			
Moisture	16.28				12.51			
Ether extract	36.39	43.47			17.80	20.35		
Crude protein	15.81	18.88	33.40		20.88	23.87	29.96	
Ash .	31.40	37.51	66.34		44.60	50.98	64.00	
Calcium	11.92	14.24	25.18	37.96	17.56	20.07	25.20	39.37
Phosphorus	5.28	6.31	11.16	16.82	7.61	8.70	10.92	17.06
Left ninth rib					Total skeleton			
Moisture ..	26.31				31.81			
Ether extract	7.82	10.61			17.18	25.20		
Crude protein .	23.31	31.63	35.39		18.93	27.76	37.10	
Ash ..	37.90	51.43	57.54		28.91	42.40	56.67	
Calcium	14.77	20.04	22.42	38.97	11.02	16.16	21.61	38.12
Phosphorus .	6.29	8.54	9.55	16.60	4.83	7.09	9.48	16.72

Ca to P ratio, tibia 2.257, ulna 2.307, rib 2.348, total skeleton 2.28.

to nutritional status. The latter is obviously difficult to assess, but the attempt to do so seems well worth while.

The age-height-weight relationship, when applied to Edwards' (11) nomogram, indicates that the subject was about 11 per cent underweight. However, as Edwards points out, actuarial records show that there are only small differences in mortality ratios (ratios of actual to expected deaths) within a range of 15 per cent above and below the average weight, so that the observed deviation of 11 per cent, if it is anything more than an error of sampling, can hardly indicate a considerable impairment in nutritional status.

An application of the nutritive index of Cowgill and Drabkin (12),

$N = W^{1/3}/L$ , in which  $W$  is the body weight in gm. and  $L$  is the stem length or sitting height in cm., leads to a value of 0.414, somewhat less than the average value of 0.45. However, the measured stem length of this subject, 99.8 cm., seems much too high for his height, 183 cm. From the DuBois data, Cowgill and Drabkin obtained the following simple expression relating height  $H$  to stem length  $S$ , both being expressed in cm.;  $S = 0.4H + 10.5$ . Applied to the cadaver analyzed, the stem length becomes 83.7 cm., 16 per cent less than that observed. The latter value is 5.7 times the average deviation of calculated stem lengths from measured ones and is highly significant statistically if the distribution of stem lengths is symmetrical. The difficulty in measuring stem length in a lifeless body may be responsible in large part for the 16 per cent discrepancy between expected and observed stem lengths. If the stem length is taken as 83.7 cm., the nutritive index becomes 0.494, being quite within the normal range.

If adequate data on well nourished human subjects were available for comparison, the water and fat content of the specimen analyzed should be indicative of general nutritional status. The total water content of 67.85 per cent is higher than many values reported in the literature, but is lower than some: the source or antecedents of most of these values are obscure. From a study of old German analyses, Albu and Neuberg (13) estimate the average water content of adult man to be 58 per cent. McQuarrie (14) in his review of water metabolism in health and disease states that "Varying somewhat with the amount of fat present, the body of the adult contains between 58 and 65 per cent water." Moleschott (15) gives the water content of a 30 year-old man weighing 63.6 kilos as 67.6 per cent. The water content of a man weighing 65 kilos is estimated by Skelton (16) from previously published German data as 63 per cent. This is practically the same as the value given by Shohl (9), 63.1 per cent. Laviates *et al.* (17) arrive at a higher value, 70 per cent, on entirely different evidence. In an unpublished experiment on forced urea feeding, the urea was found to distribute itself throughout approximately 70 per cent of the body weight of a normal subject, and in later experiments on the water exchange in humans, an assumption that the water content of the body is 70 per cent of the body weight permitted good agreement between changes in body weight predicted on this basis and the observed changes in weight. Rowntree (18) sponsors a higher water content of the adult of 75 to 80 per cent.

The fat content of 12.5 per cent for the cadaver analyzed lies about midway between the unbelievably low value of 2.5 per cent given by Moleschott (15) and Shohl's estimate (9) of 19.5 per cent. In the absence of other comparable data on man, it may not be out of order to state that Shohl's value of 19.5 per cent fat would represent a considerable degree of fattening in a fully sexed male farm animal.

The nutritional status with reference to minerals of the subject of this experiment may be assessed by considering the weight of skeleton and the composition of skeleton and selected bones. The fresh skeleton, exclusive of ligaments, weighed 10,017 gm., equivalent to 14.84 per cent of the total body weight. Volkmann's subject (see above), weighing 62.5 kilos, possessed a fresh skeleton weighing 10,164 gm., or 16.3 per cent of the body weight. Whether this skeletal weight included ligaments is not known. Scammon states: "Together, the bony and cartilaginous skeleton forms from 15 to 20 per cent of the total body weight at birth. Its post-natal growth proceeds *pari passu* with that of the body as a whole and, so far as our rather meagre statistics show, the proportions between the skeleton and body weight which exist at birth remain practically unchanged. The absolute post-natal increase in the weight of the skeleton, like that of the body as a whole, is roughly twenty-fold" (19). In a later publication from Scammon's laboratory, Wilmer (20) reports that the fresh ligamentous skeleton accounts for 17.60 per cent of the body weight of the new-born as well as of the adult.

The composition of the skeleton of our subject, and of the tibia, ulna, and rib analyzed separately, is summarized in Table II.<sup>3</sup> It will be noted that the different bones differed quite definitely in chemical composition, as Weakley and Dustman (21) found to be true for the different skeletal parts of the lower animals. This means that the composition of the skeleton cannot be estimated from that of an individual bone, as Nicholls and Nimalasuriya (6) have done, and probably others also. It is obvious that the fat and water contents of different bones vary widely, and probably that different skeletons equally well calcified may differ greatly in these respects. Comparisons of the composition of skeletal parts from different bodies, therefore, are best made on the dry, fat-free basis.

According to Huggins (22), "normal mature bone contains on the average somewhat less than half its weight of water and anything up to 25 per cent fat," these constituents varying within wide limits, depending upon the specific bone, the age, state of nutrition, and species. "The composition of the dry, fat-free matter is more uniform, being roughly 30 to 40 per cent organic and the remainder inorganic material." The bones analyzed in this experiment all fall within these very general specifications, except that some were somewhat drier and the tibia was considerably richer in fat. The skeleton of Volkmann's man contained 22.11 per cent ash as compared with 28.91 per cent for our specimen. Wolff and Kerr (23) analyzed

<sup>3</sup> Other measurements on the ulna, tibia, and rib, respectively, are as follows: maximum length, in cm., 27.85, 40.7, 30.3; minimum diameter, in cm., 1.15, 2.25, 0.55; density by water displacement (determined on the fresh intact bone with all adhering tissue removed), 1.32, 1.31, 1.50.

several bones from a case of chronic fluoride poisoning, and reported the results on the dry, fat-free (alcohol and ether-extracted) bone. The total ash on this basis was 65.01, 65.51, and 66.84 per cent for the tibia, ulna, and sixth rib, respectively, as compared with 66.34, 64.00, and 57.54 for our specimen, the last value relating to the ninth rather than the sixth rib. The percentages of calcium in the ash of the fluorotic bones were abnormally high, being 45.10, 44.68, and 43.75, respectively, and the phosphorus contents were somewhat high, also, *i.e.*, 17.60, 17.81, and 17.22.

Radasch (24) determined the content of organic matter in ether-extracted compact bone from postmortem subjects. This material contained no periosteum, cancellous tissue, or marrow. For subjects from 20 to 60 years of age, the average content of organic matter for femur, tibia, and fibula was 34.46 per cent, the content of inorganic matter thus being 65.54

TABLE III  
*Per Cent Composition of Bone Ash*

Bone	Calcium content	Phosphorus content	Ratio, Ca:P	Authority
Humerus . . .	36.67	16.01	2.29:1	Gabriel (25)
Ribs	38.80	17.41	2.24:1	Gassmann (26)
	37.09	16.95	2.19:1	Loll (27)
Humerus. . .	36.31	15.43	2.35:1	Funaoka and Shirakawa (28)
Various bones. . .	35.09	15.16	2.31:1	Klement (29)
Tibia . . .	37.96	16.82	2.26:1	This experiment
Ulna . . .	39.37	17.06	2.31:1	" "
Rib	38.97	16.60	2.35:1	" "
Entire skeleton	38.12	16.72	2.28:1	" "

per cent. The tibia described in Table II, including all parts of the bone, contained 66.34 per cent ash on the dry, fat-free basis.

Table III contains values published in the literature on the calcium and phosphorus content of the ash of human bones, in comparison with those secured on bones analyzed in this experiment.

From a consideration of all of these comparisons of measurements secured on the cadaver of this experiment with those of similar material reported in the literature, there is no reason to suspect that the specimen at our disposal was malnourished in any respect. It must be admitted, however, that comparable material is small in amount and variable in significance.

*Bearing on Biochemistry of Growth*—Growth consists of the deposition within the body of the young animal of organic and inorganic substances essential to protoplasmic functioning or to the development of an architectural structure determined by its hereditary background. These substances are derived from the food supply, either directly or by metabolic

transformation, and they are deposited within the tissue cells or in the intercellular material, the former process during extrauterine growth involving mainly increase in cell size rather than increase in cell number. The chemical reactions involved in this enlargement of a young organism according to a predetermined pattern, and the factors involved in the termination of the process when a predetermined adult size is reached, are a fascinating field of biochemistry. But its practical aspects relate to the quantitative measurement of the day to day accretions of nitrogen (protein), energy, minerals, etc., since these accretions, when measured under appropriate experimental conditions, determine the requirements of net dietary nutrients for maximum growth.

Mitchell and Curzon (8) attempted to estimate the daily accretions of calcium in the case of the growing child on the basis of admittedly inadequate data. With the information now at hand on the composition of the adult human body, another attempt will be made in this section of the paper. However, in the absence of all direct information on the composition of the human body between birth, or shortly thereafter, and maturity, the attempt will represent more an illustration of method rather than the attainment of definitive values.

The growth data of Meredith (30) for boys from 5 to 17 years,<sup>4</sup> inclusive, extrapolated at both ends of the growth period to conform to a birth weight of 3.49 kilos (31) and a weight at 20 years of 67.0 kilos, were found to be satisfactorily described by a fifth degree equation,  $W = 3.49 + 7.7876t - 1.6148t^2 + 0.1799t^3 - 0.007863t^4 + 0.0001165t^5$ , in which  $W$  is the body weight in kilos and  $t$  is the age in years. The maximum deviation of weights estimated by this equation from the observed data is -7.5 per cent at 1 year of age and all other deviations are less than 5 per cent (see Table IV). Differentiation of this equation will give the rate of growth in kilos per year at any age  $t$ .

The changing calcium content of the growing boy's body was expressed by a fourth degree equation, based upon the assumption (a) that the calcium content at birth is 0.8 per cent (32), (b) that that in the adult is 1.6 per cent, and (c) that the change from the infantile to the adult percentage occurs progressively throughout growth, but more rapidly when growth is more rapid. The equation thus derived follows:  $W = 28 + 86.828t - 16.$

<sup>4</sup> In this article, Meredith reviews the evidence in the literature and adds evidence of his own concerning secular changes in the weight and height of children. He concludes: "Altogether it appears to be reliably established that the average stature and weight of white children living in the United States and enrolled at public or private schools has increased during the last half century." It is a relief to find concrete evidence that changing food habits in this country are not undermining health, contrary to the belief frequently expressed in official circles, based upon less specific evidence and an inept use of experimental and statistical data.

$5105t^2 + 1.5625t^3 - 0.04114t^5$ , in which  $W$  is the weight of calcium in gm. in the child's body and  $t$  the age in years. Differentiation of this equation will give the rate of accretion of calcium in gm. per year. Table IV con-

TABLE IV  
*Growth of Boys in Body Weight and Calcium Content*

Age	Body weight			Calcium content†	Daily accretion			Calcium content of gains in weight
	Observed*	Calculated‡	Deviation		In body weight§	In calcium		
						Total	Per kilo body weight	
yrs.	kg.	kg.	per cent	gm.	gm.	mg.	mg.	per cent
0	3.49	3.49	0	28				
1	10.6	9.84	-7.5	100	13.9	160	16.3	1.15
2	13.7	13.9	1.5	147	8.9	105	7.6	1.18
3	16.0	16.6	3.8	179	5.9	70	4.2	1.19
4	17.6	18.4	4.5	201	4.5	53	2.9	1.18
5	19.1	20.0	4.2	219	4.3	50	2.5	1.16
6	22.0	21.7	-1.4	239	4.9	60	2.8	1.22
7	24.4	23.7	-2.8	264	6.1	79	3.3	1.30
8	27.5	26.2	-4.7	297	7.6	105	4.0	1.38
9	30.4	29.2	-3.9	341	9.1	135	4.6	1.48
10	33.3	32.8	-1.5	396	10.5	167	5.1	1.59
11	36.5	36.8	1.1	463	11.6	197	5.4	1.70
12	39.5	41.2	4.3	539	12.3	223	5.4	1.81
13	44.0	45.8	4.9	624	12.5	242	5.3	1.94
14	49.9	50.3	0.8	715	12.1	251	5.0	2.07
15	55.0	54.6	-0.7	806	11.3	249	4.6	2.20
16	59.6	58.5	-1.8	894	9.9	231	3.9	2.33
17	62.6	61.8	-1.3	973	8.8	196	3.2	2.23
18	64.5	64.4	-0.2	1035	6.1	141	2.2	2.31
19	66.0	66.3	0.5	1073	3.9	63	0.95	1.62
20	67.0	67.3	0.4	1078	1.8			

\* Growth data of Meredith (30) supplemented for ages less than 5 years and more than 17 by the earlier data of Bayley and Davis (33) and by data selected by Brody (34).

† Calculated from the equation,  $W = 3.49 + 7.7876t - 1.6148t^2 + 0.1799t^3 - 0.007863t^4 + 0.0001165t^5$ , in which  $W$  = body weight in kilos and  $t$  = age in years.

‡ Calculated from the equation,  $W = 28 + 86.828t - 16.5105t^2 + 1.5625t^3 - 0.04114t^5$ , in which  $W$  = body calcium in gm. and  $t$  = age in years.

§ Obtained by differentiation of equation given in † foot-note.

|| Obtained by differentiation of equation given in ‡ foot-note.

tains the estimated rates of calcium accretion expressed in mg. per day and in mg. per kilo of body weight. The last column of Table IV contains the estimated calcium content of the daily accretions in body weight for the different ages considered. These values are quite in line with similar values

obtained for rats, chickens, and lambs cited by Mitchell and Curzon ((8) Tables 4 and 5), taken from experiments in which animals were actually analyzed at different stages of growth.

The estimated accretions of calcium per day in the body of the growing male child listed in Table IV for boys of different ages from birth to 20 years are quite similar in absolute magnitude to the estimates of Shohl (9) obtained by an analogous method. Expressed per kilo of body weight, the values in Table IV are generally smaller because of a different age-weight relationship. Both sets of data, however, integrate to nearly the same total of 1.0 to 1.1 kilos of calcium at maturity.

The results of calcium balance experiments upon children at different stages of growth generally indicate higher daily accretions of calcium than those listed in Table IV. For example, Sherman and Lanford ((35) Table 8) have published average retentions of calcium for different age intervals, and all of them, except for the 10th to the 15th year, are considerably higher than the values in Table IV. However, they integrate at 15 years to a total, 1247 gm., larger than we have found in the adult body and equivalent to 2.3 per cent. They give also average calcium retentions compiled by Macy, probably from more recent data. This series of values integrates to a total of 1418 gm., equivalent to 2.0 per cent of the adult body weight. The calcium balances of children selected by Leitch (7) are much higher and lead to an estimate of 3.6 per cent of calcium in the adult.

The possible causes of the discrepancies between the two methods of estimating daily accretions of calcium by the growing child are discussed by Mitchell and Curzon (8). It seems fair to conclude that the gross errors of the balance method all operate to increase the estimated calcium retention. The difficulties in securing calcium balances in a growing child that represent growth requirements do not seem to be commonly realized. At any given time calcium may be retained in a bone either as a stage in its orderly development at a rate that has been unimpeded by the food supply, or as a recuperative process imposed upon normal bone growth by a prior period of inadequate food supply. The latter quota is not a legitimate part of a net requirement of calcium for bone growth, because it would not exist under conditions of continuous fully adequate nutrition, and its existence following a period of inadequate nutrition will terminate when recuperation is complete.

The way in which a bone grows would seem to confuse the interpretation of calcium balances, at least in short periods of observation. Maximow and Bloom (36), in discussing the internal reconstruction of growing bone, say: "Inside the gradually increasing mass of a growing bone, changes are constantly taking place throughout its entire period of development even to the adult stage. These consist of the formation of new lamellae by the



osteoblasts and, at the same time, of destruction and digestion of the recently formed areas of bone tissue, the formation of other new layers, and so on."

The technical errors in a calcium balance study relate mainly to a complete collection of excreta. To the extent that excreta are lost, the calcium balance is fictitiously high. No attention is ordinarily paid to the dermal loss of calcium. Bryant and Talbert (37) found calcium as a normal constituent of sweat. They reported the presence of from 5 to 10 mg. of calcium per 100 cc. of sweat, in 83 samples analyzed. Freyberg and Grant (38) were unable to detect calcium in the skin secretions of normal humans when sweating is avoided. However, in our laboratory<sup>5</sup> we have found calcium in dermal excretions under comfortable environmental conditions, equivalent to a loss of 4 to 5 mg. per hour over the entire body. Sweat always contains calcium but in highly variable concentrations from less than 1 mg. per cent to 8 or 9. Under conditions of profuse sweating, the dermal loss of calcium may range from 10 to 20 mg. per hour in normal adults. The extent of this loss in children should be studied.

The method for calcium illustrated above may be extended to protein, energy, and phosphorus. Terroine (39) has applied essentially the same method to a determination of the net protein requirements of the child. Obviously, in any consideration of nutritive requirements estimated by this or any other method, the great variability of individuals must be realized and the adaptation of the individual to a restricted food supply, as illustrated by Nicholls and Nimalasuriya (6) for calcium, cannot be neglected. Such estimated requirements are useful as guides in child feeding, but they are of little value as "yardsticks of good nutrition." It is a statistical absurdity to judge the nutritive status of large communities, even an entire population of 136 million people, by average experimental results obtained upon a mere handful of subjects, whose variation is of the order of 20 to 25 per cent.

#### SUMMARY

The chemical composition of the body of a normal adult human, 35 years of age, has been reported, with reference to moisture, ether extract, protein ( $N \times 6.25$ ), total ash, calcium, phosphorus, and gross energy. Individual analyses of the skeleton, musculature, skin, and many visceral organs are reported.

The data from this material have been considered in connection with requirements of calcium for growth on the reasonable assumption that the integration of calcium accretions during the growing period will equal the calcium content of the adult organism.

<sup>5</sup> Mitchell, H. H., and Hamilton, T. S., unpublished data.

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# THE ADSORPTION OF RIBOFLAVIN ON FLORISIL

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Since florisil was introduced by Ferrebee (1) in the determination of urinary riboflavin and applied to food assay by Conner and Straub (2), it has achieved wide-spread usage in riboflavin methodology. The efficacy of florisil for quantitative adsorption of riboflavin from natural extracts has, however, been questioned recently. In a series of collaborative assays of bread and flour, Andrews (3, 4) reported large variations in the results of the individual collaborators and indicated (4) that variations in the efficiency of different lots of florisil were partially responsible. It has been shown in this laboratory (5) that, even with florisil of a high degree of efficiency, the results on a large variety of foods and feeds still tend to be low, as judged by comparison with the results of microbiological assay of the same substances.

Two explanations have been advanced recently (6, 7) to account for the low results by florisil procedures. Hoffer, Alcock, and Geddes (6) attributed low results on flour extracts to incomplete adsorption of riboflavin *per se* on florisil and demonstrated that complete adsorption required the use of two or more successive columns of florisil. The efficiency of adsorption was shown to be dependent upon the particular lot of florisil used and also upon the concentration, volume, and clarity of the extracts from which riboflavin was adsorbed. On the other hand, Rosner, Lerner, and Cannon (7) interpreted the partial adsorption of riboflavin from extracts of natural products as being due to non-adsorption of *combined* riboflavin. Enzymic treatment resulted in complete adsorption of the riboflavin, which they interpreted as a consequence of the formation of free riboflavin.

The present report is concerned with experiments which bear on both these explanations. A study has been made of the efficiency of adsorption on florisil of (1) free riboflavin, (2) combined riboflavin (as phosphate and monosuccinate esters), and (3) naturally occurring riboflavin (in yeast and liver extract). The effects of concentration and of enzymic treatment on adsorption efficiency have also been studied. Both fluorometric and microbiological measurements have been used to determine the distribution of the riboflavin.

## EXPERIMENTAL

The florisil (30 to 60 mesh) used in all experiments was prepared as recommended by Ferrebee (1). Adsorptions were carried out in tubes which gave a column of florisil 14 cm. long by 6.5 cm. in diameter. Riboflavin extracts were prepared in 0.1 N sulfuric acid and adjusted to pH 4.5 with 2.5 M sodium acetate before adsorption on florisil. 40 ml. of 20 per cent pyridine in 2 per cent acetic acid were used for the elution of riboflavin from each column, eluates being then diluted to 50 ml. with water before fluorometry. Blank readings were taken after reduction of riboflavin with 10 to 20 mg. of solid sodium hydrosulfite. The use of the solid hydrosulfite introduced no difficulties for the types of materials examined.

TABLE I  
*Adsorption of Pure Riboflavin on Florisil*

Riboflavin put on florisil column	Volume of aliquot	Riboflavin in eluate
$\gamma$	ml.	per cent
5	20	100
5	50	100
5	100	101
10	40	100
20	40	100
25	50	100
25	100	95
50	50	98

Fluorometry was carried out in a Pfaltz and Bauer fluorophotometer, model B, fed by a special constant voltage line. The current also passes through a voltage stabilizer, insuring a high degree of reproducibility. Moreover, the use of a glass riboflavin secondary standard permitted frequent, rapid checking of instrument settings.

*Adsorption of Pure ("Free") Riboflavin*—To determine the efficiency of adsorption of pure riboflavin on the florisil used in the present experiments, graded amounts were placed on florisil at the dilutions shown in Table I. Each column was washed with 25 ml. of hot water before elution. Under these conditions, as much as 50  $\gamma$  of riboflavin were recovered quantitatively. At greater dilutions of the higher levels of riboflavin, the efficiency dropped off somewhat.

It is apparent that pure riboflavin is readily recovered quantitatively from florisil in amounts which are much greater than the 5  $\gamma$  which are placed on a column in the course of the usual assay. In all subsequent experiments, the aliquot for adsorption contained about 5  $\gamma$  of riboflavin.

*Adsorption of Combined Riboflavin*—Riboflavin phosphate and riboflavin

monosuccinate were made available to us by Mr. G. J. Haas through the kindness of Dr. M. F. Furter. The phosphate was prepared by esterification of riboflavin with phosphorus oxychloride, according to Kuhn and Rudy (8), and was about 80 per cent pure. The difficulties involved in obtaining pure riboflavin phosphate by this synthesis have been pointed out by Karrer, Frei, and Meerwein (9). The preparation was nevertheless useful in the present experiments. The succinate was prepared by esterification of riboflavin with succinic anhydride and was practically pure; traces of disuccinate may have been present.

25 mg. of each preparation were dissolved in slightly acidulated water, care being taken to avoid hydrolysis of the compounds. The solutions were buffered at pH 4.5 and further diluted. Fluorometric measurement showed the riboflavin content of the phosphate and succinate to be 79 and

TABLE II  
*Adsorption of Riboflavin Esters on Florisil*

All values are given as percentages of the initial amount of riboflavin (placed on the first column).

Compound	Adsorbed on first florisil column	In filtrate of first column	Adsorbed on second column
Riboflavin phosphate.....	86*	13	13
“ monosuccinate.....	96†	7	7

\* 25 ml. of solution containing 3.4  $\gamma$  of riboflavin were placed on the column.

† 25 ml. of solution containing 4.8  $\gamma$  of riboflavin were placed on the column.

96 per cent, respectively, of the theoretical values. 25 ml. aliquots were placed on florisil and washed with 25 ml. of hot water, with the relative results shown in Table II: the phosphate was adsorbed to the extent of 86 per cent of its riboflavin content and the succinate to the extent of 96 per cent. The filtrates (drippings from the columns, including the wash water) were passed through second florisil columns, which, as shown in Table II, completely removed the residuum of riboflavin which escaped adsorption on the first columns.

It is apparent that these riboflavin esters are adsorbed fairly completely on florisil. These data argue against the hypothesis of Rosner *et al.* (7) that combined riboflavin is not adsorbed by florisil.

*Adsorption of Naturally Occurring Riboflavin*—Two high potency materials, Fleischmann's yeast (No. 200-B) and Wilson's liver extract (Fraction B), were chosen for this phase of the work, in order that the adsorption experiments might not be overly complicated by the phenomena which affect the adsorption of low potency foods (*cf.* Hoffer *et al.* (6) on the effects

of the concentration, volume, and clarity of flour extracts on adsorption). As assayed by our fluorometric method (5), the yeast contained 79  $\gamma$  per gm. of riboflavin, while the liver extract contained 256  $\gamma$  per gm. The corresponding microbiological values, obtained by the method of Snell and Strong (10) as modified by Strong and Carpenter (11) and Bauernfeind *et al.* (12), were 75 and 257  $\gamma$  per gm.

In order to determine the effect of the *concentration* of riboflavin, extracts of both products were prepared at the extraction ratios shown in the second column of Table III, so that aliquots of 6 and 50 ml., respectively, repre-

TABLE III

*Adsorption on Florisil of Riboflavin from Extracts of Yeast and Liver Extract*

Riboflavin values are given as percentages of the amount placed on the first column of florisil.

Extract	Concentration of extract	Aliquot on first florisil column	Enzyme treatment*	Riboflavin adsorbed on first florisil column	Riboflavin in filtrate of first column	Riboflavin adsorbed on second florisil column
	<i>gm. per 100 ml.</i>	<i>ml.</i>				
Yeast	1	6	None	82	15	
		6	5% clarase	97	3	
		6	5% polidase	96	4	
	0.12	50	None	81	18	16
		50	5% clarase	82	18	15
		50	5% polidase	91	9	8
Liver	0.33	6	None	87	10	
		6	5% clarase	98	2	
		6	5% polidase	99	1	
	0.04	50	None	75	25	18
		50	5% clarase	88	11	8
		50	5% polidase	96	6	6

\* 2 hours at 45°.

sented equal weights of sample and contained approximately 5  $\gamma$  of riboflavin. The effect of enzyme treatment was also observed at both extraction ratios by comparing untreated extracts with extracts which were digested with 5 per cent clarase or 5 per cent polidase at 45° for 2 hours. 19 and 25 ml. of wash water were used for the 6 and 50 ml. aliquots, respectively, making the total volume of filtrate 25 and 75 ml., respectively.

It is apparent from the data in Table III that adsorption is more efficient from the smaller (6 ml.) aliquots than from the larger (50 ml.) aliquots, irrespective of whether or not enzyme treatment is included. Enzyme treatment did increase the efficiency of adsorption, which is practically complete for clarase and polidase in the more concentrated extracts, but

not in the dilute extracts. It was noted that digestion with these enzymes resulted in greater clarity of the extracts after filtration at pH 4.5. By comparison of the last two columns of Table III, it will be seen that most, but not all, of the riboflavin present in the filtrate of the first florisil column was adsorbed on the second column.

Microbiological assays showed that the fluorescent matter in the filtrates is actually riboflavin, good agreement being obtained between fluorometric and microbiological assays of these filtrates. It should also be noted that the sum of riboflavin adsorbed on the florisil column and the amount found in the filtrate of that column always adds up to practically 100 per cent (within the range of analytical error). As an illustration of the values

TABLE IV  
*Typical Riboflavin Balance Experiment on Yeast 200-B*

Fraction	Fluorometric		Microbiological	
	$\gamma$ per gm.	per cent*	$\gamma$ per gm.	per cent*
Yeast 200-B .....	79	100	75	95
Eluate (adsorbed).....	55	70		
Filtrate (unadsorbed).....	23	29	26.5†	33
Eluate + filtrate.....	78	99	82‡	104

\* All percentage values are based on the fluorometric value of 79  $\gamma$  per gm., which is considered 100 per cent.

† The 24 hour turbidimetric value was 26  $\gamma$  per gm. and the 72 hour titrimetric value was 27  $\gamma$  per gm.

‡ Fluorometric value for eluate used in calculating this total.

observed, a typical balance experiment in which both types of assay methods are employed is shown in Table IV.

#### DISCUSSION

It is evident from these experiments that riboflavin is adsorbed with greater ease from simple solutions than from extracts of natural substances, even when the latter are high potency sources of riboflavin, such as yeast and liver. That the efficiency of adsorption can be even poorer for low potency substances has been demonstrated by Rosner *et al.* (7) and by Hoffer *et al.* (6), and has been illustrated (5) by comparative fluorometric and microbiological assays of a large variety of low potency foods and feeds.

Rosner *et al.* (7) have sought to explain the poor adsorption by postulating that combined riboflavin is not absorbed on florisil, whereas free riboflavin is. The following considerations argue against this conception.

1. The phosphate and succinate esters used in the present experiments



were readily adsorbed on florisil (Table II). Riboflavin probably occurs in heated acid extracts of plant substances largely as free riboflavin and riboflavin phosphate. The experiment with riboflavin phosphate may therefore be considered as simulating an analytical set-up. The experiment with the succinate serves largely as a model of the behavior of another 5'-substituted ester.

2. Riboflavin which escapes adsorption on a single column of florisil can be adsorbed on successive columns. In the case of yeast and liver (Table III), practically all the riboflavin which appeared in the filtrate of the first column was adsorbed on the second column. In the case of low potency flour extracts, Hoffer *et al.* (6) showed that from one to four columns might be required, depending upon the batch of florisil and the extraction ratio, but that eventually complete adsorption can be attained.

3. Enzyme treatment does not necessarily result in complete adsorption on a single column (Table III). The fact that improved adsorption did occur in many cases after enzyme treatment can be explained equally well on the basis of the greater clarity of the extract (6) as on the basis of hydrolysis of combined to free riboflavin and adsorption of the latter (7). It should be emphasized that enzyme treatment is a desirable step in riboflavin assay.

Our experiments support the conclusions of Hoffer *et al.* (6) that, for a given lot of florisil, the efficiency of adsorption depends upon the concentration, volume, and clarity of the extracts. Since the quantitative influence of these factors varies for substances of different composition and riboflavin potency, it appears desirable to avoid the use of florisil unless it is clearly established that no losses are incurred for the particular type of sample.

#### SUMMARY

1. Riboflavin is adsorbed more efficiently by florisil from simple solutions than from extracts of high potency sources such as yeast and liver. The use of florisil in the analysis of these natural materials may cause errors ranging up to 30 per cent.

2. The factors influencing the adsorption include the clarity, volume, and concentration of the extracts.

3. Combined riboflavin, in the form of riboflavin phosphate or riboflavin monosuccinate, is readily adsorbed on florisil.

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# THE THIAMINE CONTENT OF PIG BLOOD\*

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The normal concentration of thiamine in the blood of human subjects has been determined by several investigators. In most instances an average value of the order of 8.0 to 9.0  $\gamma$  of thiamine per 100 ml. of whole blood has been found (1, 2). Goodhart and Sinclair (2) have reported a value of 5.7  $\gamma$  per 100 ml. for the ox and the considerably higher value of 20.2  $\gamma$  per 100 ml. for the pigeon. Schultz *et al.* (3) have found a value of 7.0  $\gamma$  per 100 gm. of rat blood.

Since the thiamine content of the muscle tissue of the pig is normally much higher than that for the same tissue in other species, it is reasonable to expect that the thiamine content of pig blood would also be much higher. Similarly, because the thiamine content of muscle tissue in the pig may vary according to the amount of thiamine in the diet (4), the thiamine content of the blood should be related to the thiamine content of the diet or, more directly, to the thiamine content of the muscle tissue. Information on these points is given in this report.

## EXPERIMENTAL

In order to determine the average normal concentration of thiamine, samples of blood were obtained from pigs receiving common herd rations. Some of the thiamine values represent pigs in the semifasting state, the blood samples having been taken at the time of slaughter from animals which had received no feed during the preceding 24 hours. In addition, non-fasting values were obtained from samples taken within 3 or 4 hours after the pigs had received a regular feeding. The blood samples were obtained from the anterior vena cava by the method of Carle and Dewhurst (5). Some of the blood samples were obtained from pigs fed ordinary fattening rations, the thiamine contents of which were not determined. However, most of the blood samples were taken from pigs receiving experimental diets of known thiamine content. In several instances pork samples were also obtained from these pigs.

In order to determine the effect of feeding relatively large amounts of

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thiamine upon the concentration of thiamine in the blood, daily individual doses of crystalline thiamine were administered orally to three groups of pigs. Each of a group of four pigs was given 25 mg. of thiamine for 7 days, and each pig in a similar group received 50 mg. of thiamine for the same period. Each of three pigs in a third group received 50 mg. of thiamine for a period of 11 days. Blood samples were taken before the thiamine feeding began and at regular intervals thereafter.

Thiamine was determined by a modification of the thiochrome method of Hennessy and Lewis (6). The method was modified in an attempt to reduce the hematin-catalyzed destruction of thiochrome by alkaline ferricyanide, as described by Owen, Weissmann, and Ferrebee (7). 5 ml. samples of whole, oxalated blood were pipetted into 40 ml. portions of 1 per cent acetic acid contained in 100 ml. volumetric flasks. The samples were heated at the temperature of boiling water for 10 minutes. After cooling, 0.3 gm. of taka-diastase in aqueous solution was added to each flask, and the samples were allowed to incubate at 45–50° for at least 2 hours. 40 ml. of 10 per cent trichloroacetic acid were then added to each flask, and the samples were allowed to stand for about 30 minutes. The pH was adjusted to about 4.5 with 2.5 *N* sodium acetate, the volume was made up to 100 ml., and the extracts were filtered. From this point the details of the published procedure were followed. Results were reproducible within  $\pm 15$  per cent. A moderate decrease in the destruction of thiochrome was obtained with the modified procedure.

#### DISCUSSION

The results of the investigation are summarized in Table I and Fig. 1. Although a wide range of values was encountered in several of the groups, the standard errors of the mean values are well within the limits of the experimental error.

The normal concentration of thiamine in the blood of the pig appears to be about twice as high as that reported for human subjects. The mean value found for thirteen pigs in the semifasting state was 17  $\gamma$  of thiamine per 100 ml. of whole blood. The mean blood thiamine value for nine non-fasting pigs was 21  $\gamma$  per 100 ml. The difference between these values is statistically significant and suggests that the concentration of thiamine in the blood fluctuates during the progress of digestion in a manner similar to that of blood glucose and other substances.

In general, the concentration of thiamine in the blood becomes higher as the thiamine content of the diet is increased. In addition, there appears to be a rather direct relationship between the thiamine content of the muscle tissue of the pig and the concentration of thiamine in the blood. The data for Group 7, Table I, show clearly that when the tissue stores are

large the blood thiamine level is high, even though the daily thiamine intake has been at an average level for some time. Similarly, in Group 8, although the daily thiamine intake was high, the tissue stores and the blood level of thiamine were only slightly above average. The relatively low tissue storage of thiamine in the latter animals is considered anomalous, as discussed elsewhere.<sup>1</sup>

TABLE I

*Concentration of Thiamine in Normal Pig Blood and Its Relation to Thiamine Content of Diet and to Thiamine Content of Muscle Tissue*

Group No	Description of ration	Type of blood sample	No. of animals	Thiamine per 100 ml. whole blood			Average thiamine per gm. fresh loin tissue
				Range	Mean	Standard error	
1	Herd ration of unknown thiamine content	Fasting	13	11-21	17	0.8	
2	" "	Non-fasting	5	16-21	19	0.8	
3	Herd ration containing 1.68 mg. thiamine per lb. feed	"	4	22-24	23	0.4	
4	Herd ration containing 1.75 mg. thiamine per lb. feed	Fasting	3*	16-20	18	1.2	10.7
5	Grain ration + legume pasture	"	2*	15-17	16	1.0	12.8
6	Herd ration + peanut skins or 50 mg. thiamine per day	"	4	25-35	29	2.3	19.4
7	Herd ration + 50 mg. thiamine for 35 days; then no extra thiamine for 35 days prior to slaughter	"	3	26-30	28	1.2	19.3
8	Herd ration + 50 mg. thiamine for 84 days	Non-fasting	3†	19-28	23	0.8	12.4

\* Also included in Group 1.

† A total of ten determinations on samples of 3 succeeding days.

The influence of 7 days of feeding relatively large amounts of thiamine on the thiamine content of the blood is shown in Fig. 1. The level of thiamine in the blood increased rapidly during the period of administration of the vitamin, and decreased just as rapidly after the extra thiamine was discontinued. When the vitamin was fed for a period of 11 days (results not shown), the maximum blood level values attained were no higher than those reached within 5 to 7 days.

<sup>1</sup> Pence, J. W., Miller, R. C., Dutcher, R. A., and Ziegler, P. T., unpublished data.

There are several points of difference in the results obtained in this investigation and those obtained with human subjects. Benson *et al.* (1, 8), Youmans *et al.* (9), and other workers have found that the concentration of thiamine in human blood bears little relationship to the state of saturation of the tissues. Only in cases of severe thiamine deficiency does the blood level of thiamine fall below the normal range. In a deficient human subject, the administration of thiamine will cause the blood level to rise to normal values, but in mildly deficient or normal subjects an increase in the thiamine intake will not lead to higher blood thiamine values. Najjar and Holt (10), among others, report that when relatively large amounts of

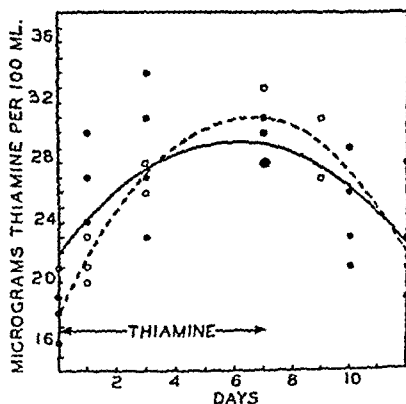


FIG. 1. The effect of feeding 25 (solid dots and line) and 50 mg. (clear circles and broken line) of crystalline thiamine daily for 7 days upon the thiamine content of pig blood.

thiamine are injected into human subjects the blood level rises to very high values, but returns to normal within a few hours.

The authors have shown<sup>1</sup> that the pig has the ability to accumulate large amounts of thiamine in muscle tissue quite rapidly. With an intake of 50 mg. of thiamine per day a maximum storage is obtained within 5 weeks, the thiamine content of these saturated tissues being nearly twice as high as that obtained from an average thiamine intake. Thus, if a high intake of thiamine is continued long enough to increase the storage of the vitamin in the muscle tissue materially, the concentration of thiamine in the blood will remain above normal even though the intake has been reduced (see the data for Group 7, Table I).

Within limitations, the concentration of thiamine in the blood may be used as a rough index of the storage of thiamine in the muscle tissue of the

pig. The storage of extra thiamine in the muscle tissue is indicated by an elevated blood thiamine value, provided that any substantial increase in the daily intake, immediately prior to sampling, has been of sufficient duration to have exerted its full effect upon the storage of this vitamin in the muscle tissue.

#### SUMMARY

The normal concentration of thiamine in the blood of pigs was found to be 17 and 21  $\gamma$  per 100 ml. for semifasting and non-fasting pigs, respectively. These values are approximately twice as high as those reported for normal human subjects.

A rise in the blood thiamine level to approximately 30  $\gamma$  per 100 ml. of whole blood occurred within a week when pure crystalline thiamine was fed in daily amounts of 25 or 50 mg.

The concentration of blood thiamine in the pig is directly related to the amount of thiamine in the diet and to the thiamine content of the muscle tissue.

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# STUDIES ON CHOLINESTERASE\*

## I. ON THE SPECIFICITY OF THE ENZYME IN NERVE TISSUE

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Recent investigations have provided evidence that the release and removal of acetylcholine is an *intracellular* process directly connected with the nerve action potential. The experiments suggest that the ester renders the neuronal surface permeable to all ions and thereby depolarizes the membrane (1-4). This concept is based mainly on studies of the enzyme cholinesterase. Three essential features are (a) the high concentration of the enzyme in nervous tissue, a concentration sufficiently high to make possible a rate of acetylcholine metabolism which parallels that of electric changes; (b) the localization of the enzyme at the neuronal surface where the bioelectrical phenomena occur; and (c) the parallelism between the enzyme activity and the voltage of the nerve action potential.

In these investigations it was assumed that the enzyme studied is a specific enzyme and that the substrate metabolized is acetylcholine (ACh). Only if this is the case is it justifiable to draw conclusions from the concentration of an enzyme as to the possible rate of the metabolism of the substrate or its function in the cell. It appears necessary, therefore, to ascertain the properties of cholinesterase and, in order to test the new concept of the rôle of ACh in the mechanism of nervous action, to establish the identity of the enzyme in the variety of tissues that were used. The results obtained are to be described in this paper and others to follow.

### Methods

The enzyme activity has been measured in the same way as described in previous papers (5, 6). The tissue is homogenized in ice-cold buffer according to the technique described by Potter and Elvehjem (7). The enzyme activity is then determined manometrically in aliquot parts of the homogenate or of the supernatant fluid after centrifugation. The buffer contains NaCl in 0.15 M, MgCl<sub>2</sub> in 0.04 M, NaHCO<sub>3</sub> in 0.025 M final concentration. Mg has been chosen as the divalent ion required for cholinesterase activity, since it is more effective than Ca (8). The total volume of the fluid in the main compartment is 3.0 ml. The substrate is placed in the

\* This work has been aided by grants of the Josiah Macy, Jr., Foundation and the Dazian Foundation for Medical Research.

side bulb in 0.1 ml., its final concentration after the mixing being about 0.015 M (see below).

The determinations with tissues of warm blooded animals were carried out at 37°, with those of cold blooded animals at room temperature (21–23°).

### Results

Cholinesterase is, by definition, the enzyme which has the function of hydrolyzing ACh. This choline ester is present in tissues, and its presence may be detected in perfusion fluids following nerve activity.

As the ester linkage in ACh shows no peculiarity, it must be assumed that it can be broken by other esterases which are widely spread in the organism. This was already pointed out by Stedman, Stedman, and Easson (9). For the same reason, it appears improbable that cholinesterase is so highly specific that no other ester linkage is attacked by it. Specificity in this case must be expected to be only relative, not absolute. Cholinesterase, in contrast to other esterases, should hydrolyze the particular substrate ACh at a higher rate than other esters, but it may split other esters as well, only at a lower rate. In order to ascertain such a relative specificity of an enzyme, it is necessary to compare the rates of hydrolysis of different esters. Only then may a picture be obtained enabling a proper distinction of the enzyme studied.

Choline esters closely related to ACh but with longer acyl chains appeared to be suitable compounds for testing the specificity. The rates of hydrolysis of propionyl- and butyrylcholine were compared to that of ACh.<sup>1</sup> Among the many other known choline esters, carbaminylocholine and acetyl- $\beta$ -methylcholine were tested, since both have pronounced pharmacological actions and are therefore of interest. Besides these choline esters, the rate of hydrolysis of benzoylcholine was studied, since it has recently been reported not to be split by brain esterase (10). The hydrolysis of all these esters by cholinesterase has been investigated repeatedly, the most complete study being that of Glick (11). The reasons why some of the previous investigations appear unsatisfactory will be discussed later.

Among the known non-choline esters, tributyrin and methyl-butyrate hydrolysis were also investigated. These two esters were first used by Stedman *et al.* (9) and later by other investigators in studies on the specificity of cholinesterase (12, 13).

*Cholinesterase in Nerve Tissue*—Those tissues were selected which were essential in the previous studies of Nachmansohn and associates in laying the basis for the new concept of the rôle of ACh in the mechanism of nervous action: (1) Mammalian brain. During the years 1937–39, it was estab-

<sup>1</sup> We are greatly indebted to Dr. H. M. Wuest of Hoffmann-La Roche, Inc., for the preparation of these compounds for these investigations.

lished that the concentration of cholinesterase in brain is sufficiently high to split significant amounts of ACh during 1 millisecond, *i.e.*, the duration of the passage of a nerve impulse (6). (2) The abdominal chain of lobster. It has been shown with these fibers which are not myelinated that the concentration of cholinesterase rises 200 to 400 per cent in those regions where synapses are located (14). (3) Nerve fibers of the squid containing the giant axon. In experiments on the giant axon of squid it has been demonstrated that cholinesterase is localized exclusively at the neuronal surface, whereas the axoplasm has practically no cholinesterase activity (15). (4) The electric organs of *Torpedo* and *Electrophorus electricus*. In experiments on these organs, a quantitative parallelism was found between the voltage of the action potential developed in these organs and the activity of cholinesterase (16, 17).

In the studies on the concentration of cholinesterase in brain, it was found that the concentration varies considerably in the different centers and different species, although remarkably constant for each center and each species (6). Therefore, not only brain tissue of several species has been used but also two tissues of the same brain but from different centers have been compared: cortex with a low and nucleus caudatus with a high concentration of cholinesterase.

Table I summarizes the data obtained. All these nerve esterases show essentially the same pattern. No substrate tested is split at a rate higher than ACh. Propionylcholine is split either at about the same or a lower rate. Butyrylcholine is split at a rate much lower than ACh or even not at all (by the enzyme of electric tissue). Of the two pharmacologically active esters, carbaminylcholine and acetyl- $\beta$ -methylcholine, the first is not split at all, the latter at a rate considerably lower than that obtained with ACh as substrate. The rate varies usually between 30 and 60 per cent of that of ACh. None of the esterases of nerve tissue splits benzoylcholine. The data obtained with carbaminylcholine are not recorded, since this compound is not split either by these or by any other esterase tested.

The non-choline esters, tributyrin and methyl butyrate, are not split by the cholinesterase of the electric tissue and of the invertebrate nerve tissue. The extract from the mammalian brain does split these esters but at a low rate. Although this is compatible with the assumption of a relative specificity as defined above, it is possible that in mammalian brain some unspecific esterase exists besides cholinesterase. Some data obtained with the enzymes of nucleus caudatus and cortex of ox are in favor of such an assumption. Tributyrin is not split by the cholinesterase of nucleus caudatus but apparently is split by that of the cortex. Such a result appears surprising, for it is difficult to assume that the properties of cholinesterase in the same brain should differ in two different centers. Since the

concentration of cholinesterase is high in the nucleus caudatus, the extract has to be diluted 10 to 20 times more than that of the cortex in order to get adequate amounts of enzyme into the manometric vessel. The lack of hydrolysis of tributyrin by the extract of the nucleus caudatus could be due

TABLE I

*Nerve Esterase; Rate of Hydrolysis of Different Substrates by Various Nerve Tissue Extracts*

(Pr = propionylcholine, Bu = butyrylcholine, Me = acetyl- $\beta$ -methylcholine (methylol), Be = benzoylcholine, Tr = tributyrin, Mb = methyl butyrate). QAc is always 100.

Species	Tissue	Equivalent weight of tissue per vessel	ACh hydrolysis; CO <sub>2</sub> in 20 min.	$Q_x = \frac{\text{c.mm. CO}_2 \text{ substrate } x}{\text{c mm. CO}_2 \text{ of substrate ACh}} \times 100$					
				QPr	QBu	QMe	QBe	QTr	QMb
		mg.	c mm.						
Rat	Brain (whole)	150	49	104	19	38	0		
		170	70	113	22	43	0		
		140	137					41	11
		140	125					47	11
Mouse	"	96	106	98	12	53	0	38	11
Ox	Nucleus caudatus	38	113	75	25	31	1	1	16
"	Cortex	104	84	94				43	25
		200	163	76	15	35	1		
Cat	Nucleus caudatus	25	122	79	14	42	0	14	5
Torpedo	Electric organ	2	117	33	0	23	0	0	0
			137	30	0	30	0	0	0
Electrophorus electricus	"**	2.7	257	97	1	22	0	0	0
Lobster	Abdominal chain	31	43	75	14	66	0		
		31	31	91	17	81	0		
		36	124					3	8
Squid	Ganglion	2	165	51	17	53	0	0	0
"	Fiber†	204	77		48			0	

\* Freshly homogenized tissue, not centrifuged.

† Containing the giant axon

to the insufficiency of the unspecific enzyme to produce an effect. This is in contrast to the cortex, in which the dilution is not as great.

In order to test this assumption, the hydrolysis of tributyrin has been measured with equal weights of homogenized nucleus caudatus and cortex of ox. In this case the rate of hydrolysis of tributyrin is exactly the same with both tissues. As an example, the data of one experiment are given: 160 c.mm. of CO<sub>2</sub> were developed in 20 minutes by 244 mg. of nucleus caudatus and 155 c.mm. of CO<sub>2</sub> by 238 mg. of cortex.

It is not impossible that with larger amounts of other nerve tissues other esterases may be detected also. This is obviously irrelevant for the problem investigated here.

*Purified and Unpurified Cholinesterase*—In view of this observation, the question may arise whether the pattern found for nerve esterases could be due, to some extent at least, to the presence of small amounts of other esterases. It appears, therefore, of interest to test the properties of a purified preparation of cholinesterase.

Such a preparation has been described by Nachmansohn and Lederer (18). The electric organ of *Torpedo marmorata* was used as material. The enzyme in this organ, as shown in the preceding paragraph, is specific cholinesterase. This is therefore the first purified preparation which can be considered as cholinesterase. Serum esterase, which was previously used as material for purification by Stedman, Stedman, and Easson (9) and later by other investigators, does not contain esterase specific for ACh (see below).

In the preparation obtained by Nachmansohn and Lederer, 1 mg. of protein was able to split 100 mg. of ACh per hour. With the electric tissue of *Electrophorus electricus* the purification of cholinesterase has been continued to a degree at which 1 mg. of protein splits 3000 mg. of ACh per hour. In order to obtain a notion of the degree of purity, it may suffice to say that in fresh horse serum 1 mg. of protein splits about 0.5 mg. of ACh in 60 minutes. The methods of purification and the physical and physico-chemical properties of this purified enzyme will be described in a separate paper.

Table II gives the rate of hydrolysis obtained with this purified preparation No. 710. Also, another preparation is described, 1 mg. of which splits 1400 mg. of ACh per hour (Preparation 217). With all substrates used the rate of hydrolysis with both preparations is identical with that obtained with the fresh homogenate recorded in Table I. There the homogenized electric tissue was put directly into the manometric vessel, in the same way as in the experiments in which the parallelism between voltage and enzyme activity was established. The identity of the two patterns is of particular significance in view of this parallelism. Since after purification to such a high degree the enzyme still shows the same properties, the results are consistent with the conclusion that the enzyme determined in these investigations is really exclusively cholinesterase. The concentration of this enzyme, therefore, may well be used as an indication of the rate of the metabolism of ACh, and the parallelism between cholinesterase concentration and voltage established becomes particularly pertinent.

*Unspecific Esterase*—In order to establish the difference between cholinesterase and other esterases, three tissues were studied in which there was no reason to assume the presence of cholinesterase other than that small

fraction due to the presence of nerves. Liver, kidney, and pancreas were selected. The data obtained are recorded in Table III.

The pattern differs distinctly from that of cholinesterase. In contrast to nerve esterase ACh is not the compound split at the highest rate. Propionyl- and butyrylcholine are split more rapidly. The kidney esterase,

TABLE II

*Purified Cholinesterase from Electric Tissue of Electrophorus electricus; Rate of Hydrolysis of Different Substrates*

Abbreviations of substrates as in Table I. 1 mg. of protein of Preparation 217 splits 1400 mg. of ACh per hour and in Preparation 710, 3000 mg. of ACh per hour.  $Q_{ACh} = 100$ .  $Q_{Be}$ ,  $Q_{Tr}$ , and  $Q_{Mb}$  are zero with both preparations.

Preparation No.	ACh hydrolysis; CO <sub>2</sub> in 20 min.	$Q_{Pr}$	$Q_{Bu}$	$Q_{Me}$
	<i>c.mm.</i>			
217	104	101	3	31
710	107	109	1	18

TABLE III

*Tissue Esterases; Rate of Hydrolysis with Different Substrates*

Abbreviations of substrates as in Table I.  $Q_{ACh} = 100$ .

Species	Tissue	Equivalent weight of tissue per vessel	ACh hy- drolysis; CO <sub>2</sub> in 20 min.	$Q_{Pr}$	$Q_{Bu}$	$Q_{Me}$	$Q_{Be}$	$Q_{Tr}$	$Q_{Mb}$
		<i>mg.</i>	<i>c.mm.</i>						
Rabbit.....	Liver	152	69	145	135	52	34		
		775	207		124		11		
		324	126	128		69		19	0
		355	159					13	3
Guinea pig.....	Pancreas	12	128	194	231	2	68	24	
		26	237		171	4	61	23	
		12	143	215					27
		10	86						40
" ".....	Kidney	102	12	270	415	0	224	149	
		264	51		372		240	68	26
		320	45	208	327	12	137		30

or esterases, splits butyrylcholine even at a higher rate than propionylcholine and about 300 to 400 per cent faster than ACh. Acetyl- $\beta$ -methylcholine is split at a low rate by the esterases present in kidney and pancreas, whereas the enzyme of the liver splits this compound at a rate comparable to that observed for cholinesterase. Benzoylcholine is split by all three enzymes; at a conspicuously high rate, however, by rat kidney extract.

Carbaminylocholine is not attacked at all, neither by cholinesterase nor by the other esterases tested thus far.

Tributyrin and methyl butyrate are hydrolyzed at a rate which is only one-fourth to one-third that of ACh. Here again are two exceptions: kidney extracts split tributyrin at a rate similar to that of ACh; rabbit liver extract seems to have little or no action on methyl butyrate.

The effects observed with different tissue esterases are not as uniform as with the cholinesterase of nerve tissues. This is not surprising. For cholinesterase ACh is the physiological substrate. The physiological substrate of the other esterases is unknown and is most likely not the same in the different organs. It appears probable that there exist a variety of

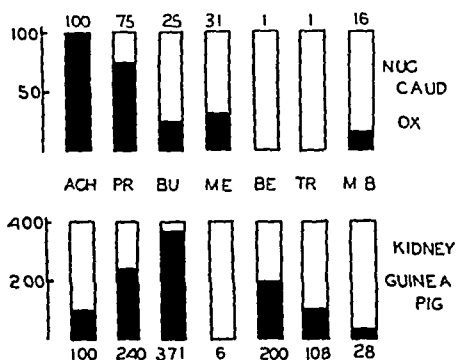


FIG. 1. Pattern of cholinesterase (nucleus caudatus of ox) in presence of different substrates compared to that of an esterase (kidney) not specific for acetylcholine. The columns represent the  $Q$  of the substrates, the  $Q$  of ACh being 100. Abbreviations are as in Table I.

esterases and possibly a mixture. All the substrates used are not physiological compounds, except ACh, and serve here only as a means of distinction. In spite of all variations, there is a general pattern which distinguishes all other esterases from cholinesterase. The use of one or two compounds is, however, not sufficient and any rigid statement should be avoided in the case of an enzyme with relative specificity. But if a number of substrates are tested, a satisfactory answer may be obtained to the question of whether or not an enzyme is specific cholinesterase. In Fig. 1 two typical patterns are reproduced which demonstrate the contrast between cholinesterase and other esterases.

*Esterases in Human Blood*—Blood serum has been used in much of the work on cholinesterase, both in specificity studies and in investigations on variations of the enzyme activity in various physiological or pathological



conditions, especially in disorders of nervous function. Stedman *et al.* (9) found that horse serum, after purification by means of ammonium sulfate precipitation, changes its ratio of activity towards esters of simple alcohols and choline esters respectively, the latter being split at a relatively higher rate by the purified enzyme. Butyrylcholine was split by both preparations at a higher rate than ACh. The authors considered this purified preparation a specific cholinesterase and different from the enzyme splitting aliphatic esters. The existence of two different esterases has been confirmed by Richter and Croft (13). Using a great variety of choline esters and other substrates, Glick (11) tested the specificity of cholinesterase on unpurified horse serum and arrived at the conclusion that the enzyme tested is unspecific.

TABLE IV

*Esterases in Human Blood Serum and Red Blood Cells; Rate of Hydrolysis with Different Substrates*

Abbreviations as in Table I. The serum was diluted about fifteen to thirty times, the hemolyzed red blood cells about 50 times.  $Q_{ACh} = 100$ .

Material	ACh hydrolysis; CO <sub>2</sub> in 20 min.	Q <sub>Pr</sub>	Q <sub>Bu</sub>	Q <sub>Me</sub>	Q <sub>Bc</sub>	Q <sub>Tr</sub>	Q <sub>Nb</sub>
	<i>c.mm.</i>						
Serum.....	181	191	279		68	90	
	115	175	266	0	52	106	9
	264			4			6
Red blood cells .....	128	87		39		11	9
	117		9		5		
	159	76	7	38	2	18	6

In human plasma Vahlquist (12) was unable to separate the esterase splitting ACh and the aliphatic esters. He concluded from his observations that in human plasma the same enzyme is responsible for the hydrolysis of aliphatic and choline esters and that there is no evidence for a specificity of this enzyme towards ACh. Applying different kinds of evidence, Easson and Stedman (19) and Richter and Croft (13) all arrived at the same conclusion, that in human serum only one esterase is present.

In Table IV are recorded the data obtained with human serum by use of the different substrates to distinguish between specific and unspecific esterases. The pattern obtained is that of the enzymes not specific for ACh. This is in agreement with the conclusion of Vahlquist.

Alles and Hawes have provided evidence that the esterase present in human red blood cells differs from that in serum in several respects (20).

According to Richter and Croft the cholinesterase of human blood cells is completely specific in its action on ACh, the activity towards tributyrin and methyl butyrate being less than 3 per cent of that towards ACh, in contrast to the serum esterase. The figures recorded in Table IV show that the hydrolysis pattern of red blood cells is similar to that of cholinesterase, although with some small, probably insignificant, deviations. This specificity is in agreement with the conclusion of Richter and Croft. The physiological significance is at present not clear.

*Muscle Esterase*—It is possible that propagation of an impulse in muscle is similar in nature to that in nerve fiber. It is therefore of interest to know whether the esterase present in muscle is cholinesterase.

Nerve-free parts of cat and rabbit gastrocnemius have been used as material. If only slices near the upper and lower surfaces of the middle

TABLE V

*Esterase in Striated and Heart Muscle; Rate of Hydrolysis with Different Substrates*

Abbreviations as in Table I. The tissue is homogenized, but not centrifuged.  $Q_{ACh} = 100$ . Benzoylcholine is not hydrolyzed by any of these preparations.

Species	Muscle	Equivalent weight of tissue per vessel	ACh hydrolysis; $CO_2$ in 90 min.	$Q_{Bu}$	$Q_{Me}$
		mg.	c mm.		
Cat ..	Gastrocnemius (nerve-free)	376	60	2	27
Rabbit	" " "	376	89	15	49
Ox	Heart (apex)	310	64	41	21
		320	78	33	40

part of the interior section are taken, the muscle tissue is practically free of nerves and nerve endings, as shown previously (21). Microscopic controls were made. The data obtained indicate that the enzyme is cholinesterase (see Table V). This is also the case with esterase present in the apex of ox heart muscle.

The concentration of cholinesterase in muscle fibers is low (22), but the conducting surface per unit of weight is small. As repeatedly emphasized, the presence of a specific enzyme alone does not permit any conclusion as to its function; only in connection with other facts may an interpretation become possible. But it permits the assumption that the substrate has a function in the cell, whereas the absence of the enzyme would make such an assumption unlikely.

*Optimal Substrate Concentration of Cholinesterase*—Marnay and Nachmansohn found, in 1937, the optimal concentration of ACh for the manometric determination to be close to  $10^{-2}$  M (22). In controls carried out

repeatedly since then with a great variety of nerve tissues, this substrate concentration was always found to be close to the optimum. With lower substrate concentrations the rate of hydrolysis decreases rapidly. If the rates are plotted against the logarithm of the molar concentration, an S-shaped curve is obtained. This is in agreement with the observations of Michaelis and Menten on the interrelationship between enzyme activity and substrate concentration (23). With a concentration higher than  $2 \times 10^{-2} M$  the rate of hydrolysis decreases. This depressing effect, with sub-

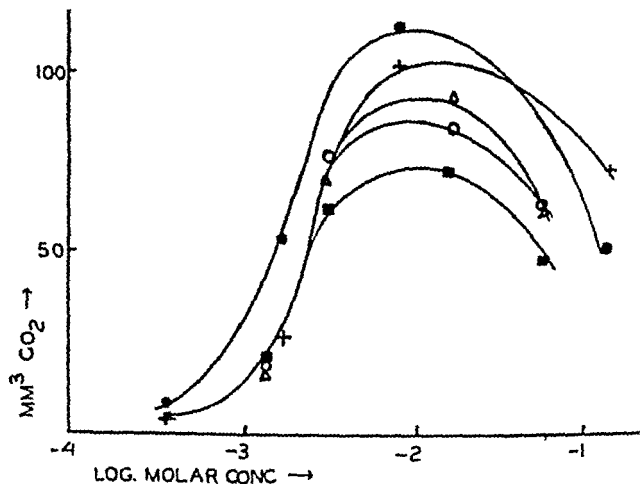


FIG. 2. Rate of hydrolysis of ACh by cholinesterase of various nerve tissues as a function of substrate concentration. Ordinates, c.mm. of  $CO_2$  liberated in 15 or 20 minutes; abscissae, log of molar concentration of ACh. O whole brain (mouse), ■ cortex (ox),  $\Delta$  nucleus caudatus (ox), + squid ganglion, ● Preparation 217. Purified enzyme from electric organ.

strate concentration above the optimum, has already been observed in the case of other enzymes. The values obtained for the rate of ACh hydrolysis by cholinesterase of various nerve tissues as a function of substrate concentration are recorded in Fig. 2.

The depressing effect with concentrations above the optimum seems to be less pronounced and not regular if other esterases are used. Fig. 3 shows that the pancreas esterase activity is not depressed and even increases slightly at the highest concentration used. Liver esterase, on the other hand, is depressed, although less markedly than cholinesterase. A marked difference is also observed between serum esterase and cholinesterase of the red blood cells, as shown in Fig. 4. This has been described by Alles and Hawes (20). Their values obtained with red blood cells are close to those

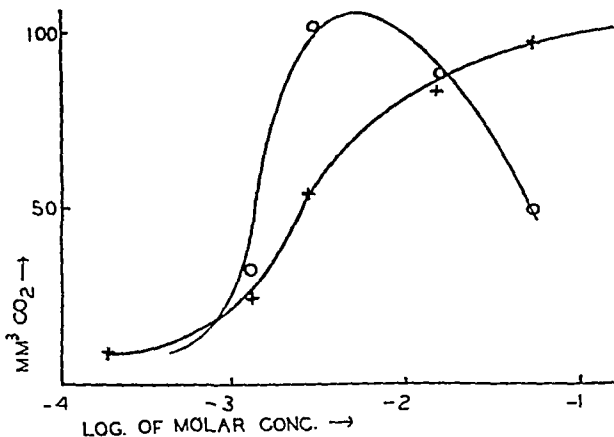


FIG. 3. Rate of hydrolysis of acetylcholine by blood esterases as function of substrate concentration. Abscissae and ordinates are as in Fig. 2. O red blood cells; + serum.

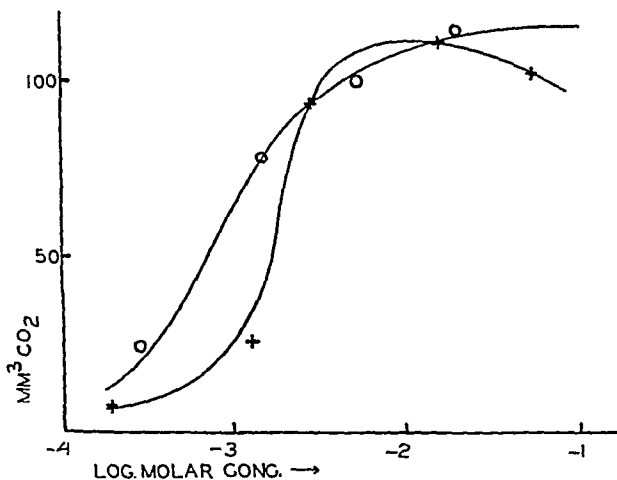


FIG. 4. Rate of hydrolysis by tissue esterases (rabbit liver and guinea pig pancreas) as function of substrate concentration. Abscissae and ordinates are as in Fig. 2. O pancreas; + liver.

recorded here. In the case of serum esterase, the rate of hydrolysis as a function of substrate concentration increases more rapidly in the experiments described here. The difference may, however, be due to the

fact that not only the method used but also the experimental conditions differ in several respects.

In these observations, the enzyme concentration is adequate for the manometric method used and is therefore always of the same order of magnitude. In most cases about 60 to 120 c.mm. of  $\text{CO}_2$  are liberated in 15 to 20 minutes with optimal substrate concentration. With other methods, a different enzyme concentration may be more adequate and may lead to a shift of the optimal substrate concentration.

Mendel and Rudney (24) recently claimed to have evidence for the existence of a pseudocholinesterase as distinguished from the true cholinesterase. According to these authors, there is "a decisive difference between the two esterases, calling for a sharp distinction." The pseudocholinesterase exhibits its maximum activity at high concentration of ACh (above 300 mg. per 100 ml. =  $2 \times 10^{-2}$  M); the true cholinesterase displays its maximum activity at low substrate concentrations (below 3 mg. per 100 ml. =  $2 \times 10^{-4}$  M). These results of Mendel and Rudney are in contrast to the facts described here and to all previous results of Nachmansohn and associates. They appear to be due to the adoption of conditions unsuitable for the method used; the values for the rate of hydrolysis by the true cholinesterase are obtained in a 2 minute period after addition of the substrate to the enzyme ((24) Table VII). During that period thermal equilibrium is not yet reestablished and the values are meaningless. This difference of enzyme activity at low and high concentrations, which is the essential distinction between "pseudo" and "true" cholinesterase, thus seems to be baseless.

#### DISCUSSION

The most important result of the data presented is the evidence that the enzyme in the various nerve tissues on which the physiological rôle of ACh was studied is a specific cholinesterase. Thus, the conclusion appears justified that the enzyme activities determined may be used as an indication of the ACh metabolism. The observations therefore lend further essential support to the new concept of the rôle which ACh may have in the mechanism of nervous action.

This concept has been criticized by Glick (25) as being a "theoretical structure" on the ground that there does not exist a specific cholinesterase and that, therefore, the enzyme activities studied do not permit any conclusion concerning ACh metabolism. Glick's statement is based on his specificity studies in which unpurified horse serum was used. From the results described by previous investigators, no specificity could be expected in that case. The rates obtained with the different substrates for the esterase in human serum described above are in agreement with those ob-

tained by Glick with horse serum. But it is difficult to see what connection this material should have with the mechanism of nervous action. Since, according to the new concept, the release and removal of ACh are considered as an *intracellular* process, only the esterase present in nerve tissue is of interest.

It is remarkable that the cholinesterase in all nerves is not only specific for ACh but seems to have similar properties, whatever the source of the material may be, mammalian brain or invertebrate nerve or electric organ of fish. It is, of course, possible that with a greater variety of substrates some difference may be detected. Other properties, among them, for instance, the action of ions or the affinity to drugs, may also vary according to the material used. The study of these problems is being carried out and will be described in papers to follow. The patterns obtained are sufficient to warrant the special nature of the esterase of nerves, in which ACh is known to be the substrate, in contrast to the esterases in other tissues in which the substrates are unknown.

#### SUMMARY

1. By testing the action of an esterase on a number of substrates, a pattern may be obtained which makes it possible to distinguish the specific cholinesterase from other esterases. The main feature of cholinesterase is the fact that no other substrate is split at a higher rate than ACh. If propionylcholine is the substrate used, the rate may still be the same; sometimes it is lower. With butyrylcholine it is always markedly lower or sometimes near to zero. Acetyl- $\beta$ -methylcholine is split at a lower rate, carbamylcholine and benzoylcholine not at all. Esters of simple alcohols are hydrolyzed either at a low rate or not at all.

2. The other esterases probably present a variety of enzymes and their pattern is therefore less uniform. But all show definite differences if compared with cholinesterase and may hereby be easily distinguished. ACh is not the compound which is split at the highest rate.

3. The esterase in all nerve tissues is either exclusively or predominantly cholinesterase. Those nerve tissues have been tested which in previous studies had provided evidence for a new concept of the rôle of ACh in the mechanism of nervous action: mammalian brains of different species, abdominal chain of lobster, squid ganglion and axon, and electric tissue of fish.

4. No difference is found between the properties of the esterase of freshly homogenized electric tissue and that obtained after purification to a high degree. This supports the view that the parallelism between voltage and enzyme activity observed in electric fish indicates a parallelism between voltage and ACh metabolism.

5. Observations of previous investigators are confirmed that the esterase in human serum is unspecific, and that in the red blood cells is specific for ACh.

6. The esterases in striated and heart muscle both free of nerve endings show a pattern typical of cholinesterase.

7. Some observations are described on the rate of hydrolysis as a function of substrate concentration.

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# STUDIES ON BODY COMPOSITION

## I. THE DETERMINATION OF TOTAL BODY FAT BY MEANS OF THE BODY SPECIFIC GRAVITY\*

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Recent investigations by Behnke (1) on the physical fitness of military personnel have emphasized the importance of fat in body composition and function, particularly in relation to the phenomenon of "bends." Furthermore, the importance of expressing the various tissue components and functions on a fat-free basis has been stressed by many workers, *e.g.* Hastings (2).

In view of the low density of fat when contrasted with that of the other body constituents, some relationship between the amount of fat in an individual and his whole body specific gravity is to be expected. Methods for determining corporeal specific gravity are described by Bohnenkamp and Schmäh (3), Jongbloed and Noyons (4), and Behnke, Feen, and Welham (5). The workers last named consider body density an index of obesity. Their method is based upon a water displacement technique and appears to be the most simple and reproducible thus far advanced for measuring body density in man.

Tester (6) has shown that the relationship of the oil content (ether extract) to a factor based on body specific gravity is linear in herring. Up to the present, however, there have been no simultaneous analyses relating the body fat content of mammals to their body specific gravity.

It is apparent that the specific gravity method of determining whole body fat requires experimental validation with gravimetric fat analysis. We have attempted to do this using guinea pigs, the specific gravity of which was determined by water displacement and the fat content by petroleum ether extraction.

### EXPERIMENTAL

50 normal male and female guinea pigs with wide variation in weight and fat content were selected from stock animals and killed by a blow on the

\* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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head. The viscera were dissected out, leaving only the kidneys and genital organs with surrounding fat. The hair was removed with animal clippers.

Specific gravity was determined on the eviscerated animal by the water displacement method. A Harvard trip balance was supported over a tank of water and the carcass was weighed in air to the nearest 0.1 gm. By means of a long hook attached under the balance the carcass was suspended and weighed under water. Care was taken that no bubbles of air were trapped in the body. The difference in weight was equivalent to the number of cc. of water displaced, and the specific gravity of the animal could be calculated readily according to the formula, specific gravity = (weight in air)/(weight in air minus weight in water). No attempt was made to correct for the density change of water with temperature, as this proved to be negligible.

The complete carcass, including skin, was chopped with a cleaver into pieces approximately 1 inch square. This was accomplished more easily when the tissue was chilled; so before and after chopping, the tissue was kept in a refrigerator at  $-8^{\circ}$ . Some drying of the tissue also resulted from this step.

The pieces of tissue were next spread in layers in vacuum desiccators, and the latter were attached to a Stokes cryochem apparatus. By this means the tissue was dried under a vacuum and below room temperature for 48 hours.

The presence of much body fat gave a soft oily appearance to the tissue even though practically all the water had been removed, and made further subdivision of the tissue difficult. Consequently, when the body specific gravity was found to be below 1.080, some fat was preextracted from the dried tissue pieces by allowing them to stand under ether overnight, and the extracted tissue residue was redried and weighed. The weight of the fat extracted was noted and considered in the final percentage. This preextraction of the tissue pieces facilitated their pulverization as described below.

Attempts at drying the original tissue pieces to constant weight proved unsatisfactory for dry weight determination. Therefore, the dry weight values were reckoned as the sum of the fat-free dry weight plus the total weight of extracted fat. The nine leanest animals in the series (*i.e.*, those whose specific gravity was above 1.080) were not crudely preextracted, and the water percentages on these animals were somewhat low because the tissue apparently could not be dried as completely as that which had been preextracted.

The dried guinea pig tissue, which included the entire skin and skeleton, could be ground into a homogeneous powder by putting the dried tissue pieces through a Wiley laboratory mill equipped with a 2 mm. mesh sieve, and thoroughly mixing the product. Thus, it was possible to use aliquot

samples of the entire carcass for the fat analysis. Readily reproducible values for fat content could be obtained on dried powder samples of 0.2 to 0.5 gm.

Continuous fat extraction was carried out in a Soxhlet apparatus for 24 hours with petroleum ether (b.p. 35–60°) as the solvent. The fat was determined by the difference in weight of the tissue powder before and after extraction. Small bags of fiberglas cloth were used to hold the powder and these permitted final drying, weighing, extracting, and reweighing without transfer of the sample, as well as the simultaneous extraction of several samples in one Soxhlet chamber.

The cloth and thread from which the bags were made were obtained from the Owens-Corning Fiberglas Corporation. The bags were approximately 1 inch square, stitched on three sides, with a lucite solution applied to the cut surfaces to prevent fraying; the open end was closed with a flap which was folded over and fastened with a fine wire. This same wire was used to suspend the bags from small racks for drying the tissue powder in a desiccator.

The fiberglas material was not hygroscopic and the bags could be dried readily to constant weight. The desiccated tissue was found to be somewhat hygroscopic, but with reasonable care and speed in weighing reproducible weights could be obtained.

On twenty-one of the guinea pigs in the series the specific gravity and fat content of the viscera, as well as of the eviscerated carcass, were determined. The viscera were rinsed thoroughly on removal and the entire intestinal tract, including the stomach and cecum, was emptied and cleaned. The lungs were discarded, since attempts to evacuate them were unsuccessful. The specific gravity of the viscera was determined by the water displacement technique, after which they were dried and analyzed for fat content as above.

#### RESULTS AND DISCUSSION

It may be noted from original data taken on the 50 guinea pigs (Table I) that the range of specific gravity measurements is from 1.021 to 1.096. These values correspond with the extremes noted by Behnke *et al.* (5) for man.

The results of the fat analyses show a range of 1.5 to 35.8 per cent of the wet weight of the eviscerated carcass. When calculated as the percentage of the dry carcass weight, the range is from 5.0 to 66.8 per cent.

The possible discrepancy introduced by determining specific gravity and fat content on eviscerated animals was checked by analyzing separately the eviscerated carcass and the viscera of twenty-one animals and calculating the combined values. The data are plotted in Fig. 1.

From Fig. 1 it may be seen that the fat content of whole animal is

TABLE I

*Original Data on 50 Guinea Pigs Taken from Laboratory Stock*

Animal No.	Sex	Live weight	Eviscerated, wet weight	Eviscerated, dry weight	Sp gr	Total fat
		<i>gm</i>	<i>gm.</i>	<i>gm.</i>		<i>gm.</i>
79	M.	427	292.5	86.0	1.093	4.3
87	"	720	496.5	177.5	1.071	58.0
88	"	712	495.7	162.2	1.076	40.9
89	"	775	574.0	189.4	1.075	51.1
90	"	862	653.5	237.6	1.067	87.0
91	F.	863	558.2	165.4	1.075	35.4
92	M.	619	433.2	144.8	1.072	40.1
93	F.	1000	689.0	319.5	1.037	189.5
99	M.	950	700.0	294.7	1.048	146.1
100	"	894	664.5	277.0	1.047	137.5
101	"	992	755.0	303.3	1.045	141.5
102	"	987	735.0	343.0	1.033	187.1
105	"	590	423.8	142.6	1.064	40.9
106	F.	495	337.3	120.1	1.061	41.1
107	"	661	435.8	139.9	1.070	36.8
108	"	439	360.5	126.9	1.063	38.8
109	"	538	391.8	136.1	1.065	40.7
114	M.	515	404.7	143.8	1.083	17.7
115	F.	379	252.0	90.9	1.096	5.1
116	M.	415	319.0	120.3	1.074	31.4
117	"	528	393.0	138.5	1.068	32.8
118	"	483	335.3	116.8	1.086	18.5
119	"	418	284.7	94.0	1.087	6.9
120	F.	450	275.4	96.3	1.080	14.0
136	M	558	429.2	134.5	1.071	30.1
137	"	700	621.6	207.4	1.069	57.6
138	"	327	228.6	73.4	1.083	11.1
139	"	722	655.8	279.7	1.050	140.6
140	F.	617	429.6	147.0	1.067	50.9
141	M.	687	491.4	166.2	1.074	52.4
142	F.	493	356.6	112.4	1.069	34.6
143	M.	304	224.2	74.1	1.088	7.3
144	F.	646	474.2	160.2	1.062	56.2
150	M.	659	526.3	196.8	1.063	70.4
151	"	393	305.8	100.8	1.066	27.5
152	F.	475	359.8	115.4	1.081	26.5
153	M.	577	452.8	153.5	1.073	41.7
154	"	886	651.0	237.3	1.070	84.5
155	"	775	580.0	224.9	1.065	91.6
156	"	649	493.5	166.7	1.077	45.4
157	"	620	478.4	165.2	1.073	38.9
158	F.	449	291.9	93.7	1.074	24.2
159	"	660	480.8	178.3	1.059	75.1

TABLE I—*Concluded*

Animal No.	Sex	Live weight	Eviscerated, wet weight	Eviscerated, dry weight	Sp. gr.	Total fat
		gm.	gm.	gm.		gm.
160	M.	496	346.9	115.4	1.072	33.2
161	F.	542	378.2	135.1	1.062	49.9
162	"	933	750.0	391.6	1.023	258.0
163	"	800	612.2	277.2	1.039	157.2
164	M.	907	747.0	371.5	1.031	229.3
165	F.	986	768.2	411.8	1.021	275.0
166	M.	690	539.0	212.0	1.073	69.0

equivalent to that of the eviscerated carcass over the entire range. This indicates that fat is probably laid down proportionately in the viscera compared with the remainder of the body.

The case is slightly different with respect to specific gravity. It may be seen from Fig. 1 that the slope of the line representing the relationship of the whole animal specific gravity to that of the eviscerated carcass is not quite unity. This is to be expected, since the specific gravity of the viscera is less than that of the whole body. However, as the fat content increases the difference is lessened and becomes quite imperceptible. In any case the discrepancy is slight, owing to the small mass of viscera relative to the carcass, and in the lower range of specific gravity the values on eviscerated carcass may be taken as representative of the whole animal. This question is further discussed below.

It should also be pointed out that the hair was clipped from the animals and discarded before any measurements except live weight were made. Hair was found to represent about 14 per cent of the original live weight and must be considered in any *in vivo* study on fur-bearing animals. The hair was removed for several reasons. In the first place, it tended to trap air bubbles when the carcass was being weighed under water, secondly, the quantity of hair varied from animal to animal, and thirdly, the clipped animal was more comparable to man.

The relationship between total body fat per unit body weight and body specific gravity is expressed by Fig. 2. As stated by Behnke (1), variations in body fat appear to be the chief determinant of body specific gravity.

It is evident that the body specific gravity increases as the fat content decreases, and it can be shown (Paper II of this series (7)) that this is an inverse relationship; consequently, a plot as in Fig. 2 may be expected to represent the scatter about a rectangular hyperbola displaced along the principal  $y$  axis.

A straight line fit was made by the method of least squares to the experi-

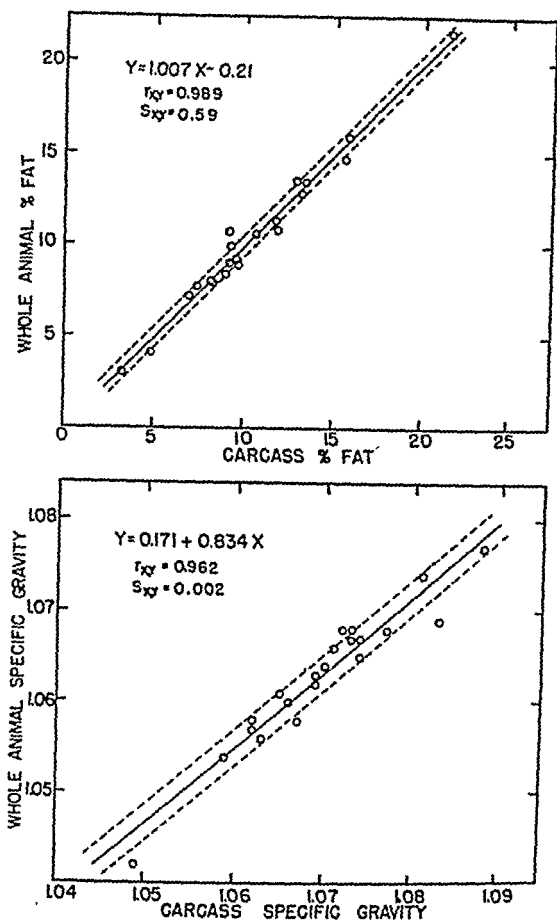


FIG. 1. The upper graph represents the per cent fat of the whole guinea pig plotted against the per cent fat of the eviscerated guinea pig. The lower graph represents the specific gravity of the whole guinea pig plotted against the specific gravity of the eviscerated guinea pig. The lines were fitted by the method of least squares.

mental values of fat content *versus* the inverse of the specific gravity and the equation

$$\% \text{ fat} = 100 \left( \frac{5.135}{\text{sp. gr.}} - 4.694 \right) \quad (1)$$

represents the least squares hyperbola through the data in Fig. 2. The correlation coefficient was computed to be  $-0.972$ , and the standard error of  $y$  on  $x$  was  $0.0187$ , or  $1.87$  per cent in fat content.

A theoretical derivation of the relationship between body fat and body specific gravity for the eviscerated guinea pig has been developed (7), and the curve represented by the theoretical equation also appears in Fig. 2. The equation for this curve is

$$\% \text{ fat} = 100 \left( \frac{5.362}{\text{sp. gr.}} - 4.880 \right) \quad (2)$$

It should be noted that the experimental curve is shifted to the left from the theoretical curve by somewhat more than the standard error of experi-

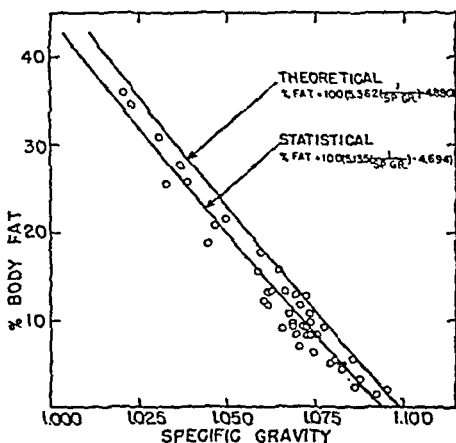


FIG. 2. The gm. of fat per 100 gm. of eviscerated body weight are plotted against the body specific gravity for the guinea pig. The equations for the lines were obtained as explained in the text.

ment. This may be due to a small undetected constant error in the present specific gravity procedure.

To correct for the slight discrepancy which exists between specific gravity of the eviscerated animal and that of the whole animal, a theoretical equation for the whole guinea pig has also been derived (7) and may be written

$$\% \text{ fat} = 100 \left( \frac{5.501}{\text{sp. gr.}} - 5.031 \right) \quad (3)$$

A comparison of the fat content, given in Table I, of the male guinea pigs with that of the female guinea pigs was made on a fat-free body wet weight basis. The seven largest males, on the fat-free basis, were omitted in order to make the comparison over the same range of weights. The mean fat-free weight for the females was 378.9 gm., and for the males 388.8 gm. The mean fat content for the females was found to be 14.5 per cent,

and that for the males 9.8 per cent. The difference was statistically significant, and appeared to be greater with the larger animals; *i.e.*, the larger females tend to contain more fat in proportion to the corresponding males than in the smaller size group.

In view of the fact that the specific gravity range for guinea pig and man matches rather well, and because of the probably relatively small variation

TABLE II

*Conversion of Values for Body Specific Gravity of Man to Fat Content on Basis of Equation, Per Cent Fat = 100 (5.548/(Specific Gravity) Minus 5.044)*

Body sp. gr.	Per cent fat of body weight	Body sp. gr.	Per cent fat of body weight
1.002	49.3	1.052	23.0
1.004	48.2	1.054	22.0
1.006	47.1	1.056	21.0
1.008	46.0	1.058	20.0
1.010	44.9	1.060	19.0
1.012	43.8	1.062	18.0
1.014	42.7	1.064	17.0
1.016	41.7	1.066	16.1
1.018	40.6	1.068	15.1
1.020	39.5	1.070	14.1
1.022	38.5	1.072	13.1
1.024	37.4	1.074	12.2
1.026	36.3	1.076	11.2
1.028	35.3	1.078	10.3
1.030	34.2	1.080	9.3
1.032	33.2	1.082	8.4
1.034	32.2	1.084	7.4
1.036	31.1	1.086	6.5
1.038	30.1	1.088	5.5
1.040	29.1	1.090	4.6
1.042	28.0	1.092	3.7
1.044	27.0	1.094	2.7
1.046	26.0	1.096	1.8
1.048	25.0	1.098	0.9
1.050	24.0	1.100	0.0

in the ratios of tissue components aside from fat in man as well as in the guinea pig, it appears possible to apply an equation similar to Equation 3 for estimation *in vivo* of total body fat in man.

Human fat has a density of 0.918 (Hodgman (8)), and from the data of Behnke *et al.* (5) a value of 1.10 may be estimated for the density of the fat-free human body. With these values as limits in the basic equations given in (7) it is possible to derive a provisional equation for the conversion of

human body specific gravity to the corresponding fat percentage. This equation is found to be

$$\% \text{ fat} = 100 \left( \frac{5.548}{\text{sp. gr.}} - 5.044 \right) \quad (4)$$

Thus direct substitution in Equation 4 of values for body specific gravity of man obtained by the method of Behnke, Feen, and Welham (5) would yield values for total body fat.

By means of Equation 4, Table II has been constructed, from which values of fat content may be read for body specific gravity of man in the range of 1.002 to 1.100. As mentioned previously, Behnke *et al.* (5), in their study of the body specific gravity of 175 naval personnel, found a range of 1.097 to 1.021 for the group. From Table II it may be seen that this represents a range of fat content of 1.4 per cent to 39.0 per cent of the body weight. It is also of interest to note that Behnke *et al.* divided the subjects into three groups on the basis of the specific gravity, these being less than 1.060, from 1.060 to 1.074, and above 1.074. The corresponding fat contents are respectively greater than 19.0 per cent, from 19.0 per cent to 12.2 per cent, and less than 12.2 per cent.

It is with considerable pleasure that we acknowledge the kind suggestions and great interest of Captain A. R. Behnke (MC) U. S. N., during the entire course of the work, and also the generous assistance of Lieutenant (j.g.) Manuel F. Morales, H(S), U. S. N. R., in the preparation of the manuscript.

#### SUMMARY

1. Methods are described for the determination of body specific gravity of guinea pigs by water displacement and their total body fat content.

2. The data are presented for a series of 50 normal animals for which specific gravity of the eviscerated body ranged from 1.021 to 1.096, and whose fat content varied from 1.5 to 35.8 per cent of the body weight.

3. The experimental relationship between eviscerated body specific gravity of the guinea pig and body fat was found to agree closely with a theoretically derived relationship which may be expressed by the equation,

$$\% \text{ fat} = 100 \left( \frac{5.362}{\text{sp. gr.}} - 4.880 \right)$$

4. A sex difference in fat content is demonstrable when the animals are compared on a fat-free body weight basis. The female guinea pigs averaged 4.7 per cent more fat than the males.



5. It appears possible to apply an equation similar to that for the guinea pig for estimates of total body fat in man. The equation is

$$\% \text{ fat} = 100 \left( \frac{5.548}{\text{sp. gr.}} - 5.044 \right)$$

Table II, based on this equation, is also given for the conversion of specific gravity of man to per cent body fat.

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# STUDIES ON BODY COMPOSITION

## II. THEORETICAL CONSIDERATIONS REGARDING THE MAJOR BODY TISSUE COMPONENTS, WITH SUGGESTIONS FOR APPLICATION TO MAN\*

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The mammalian body can be regarded as consisting of certain major phases: fat, bone, muscle, skin, nervous and visceral tissue. To be sure there are heterogeneities within each phase, but these are negligible in comparison to the differences in individual properties of the phases. Physiological experiments on intact animals frequently depend for their success on knowledge of how much of each of these components is present. To measure the relative proportion in the intact animal requires an indirect method. The present work is concerned with the theory and use of one such method.

*Theoretical Considerations*—It is assumed (1) that the fat component is the one subject to wide variation, while the proportions of other components to some standard component, say bone, are relatively constant (2).

In terms of the following notation,

$M_f$ and $D_f$	=	mass and density respectively	of	fat
$M_b$ and $D_b$	=	" " "	"	" bone
$M_m$ and $D_m$	=	" " "	"	" muscle
$M_s$ and $D_s$	=	" " "	"	" skin
$M_n$ and $D_n$	=	" " "	"	" nervous tissue
$W$	=	weight of animal		

and it is assumed that (a) muscle, skin, and nervous tissue are (in the adult) constant fractions,  $k$ , of the mass of bone, or, symbolically,

$$M_m = k_m M_b \quad (1)$$

$$M_s = k_s M_b \quad (2)$$

$$M_n = k_n M_b \quad (3)$$

\* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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and that (b) the densities of skin and nervous tissue are approximately the same as that of muscle:

$$D_s \cong D_n \cong D_m \quad (4)$$

From the notation and Assumptions 1 to 4, the following equations are obtained: the *total mass* of the body is

$$W = M_f + M_b + M_m + M_s + M_n = M_f + M_b(1 + k_m + k_s + k_n) \quad (5)$$

and the *average density*,  $G$ , of the body is

$$G = \frac{W}{\frac{M_f}{D_f} + \frac{M_b}{D_b} + \frac{M_m}{D_m} + \frac{M_s}{D_s} + \frac{M_n}{D_n}} = \frac{W}{\frac{M_f}{D_f} + M_b \left( \frac{1}{D_b} + \frac{k_m + k_s + k_n}{D_m} \right)} \quad (6)$$

If  $k_m + k_s + k_n = K$ , Equations 5 and 6 are readily solved for

$$\frac{M_f}{W} = \frac{\left( \frac{1 + K}{G} - \frac{D_m + KD_b}{D_b D_m} \right)}{\left( \frac{1 + K}{D_f} - \frac{D_m + KD_b}{D_b D_m} \right)} \quad (7)$$

$$\frac{M_b}{W} = \frac{1}{1 + K} \left( 1 - \frac{M_f}{W} \right) \quad (8)$$

$$\frac{M_m}{W} = \frac{k_m}{1 + K} \left( 1 - \frac{M_f}{W} \right) \quad (9)$$

$$\frac{M_s}{W} = \frac{k_s}{1 + K} \left( 1 - \frac{M_f}{W} \right) \quad (10)$$

$$\frac{M_n}{W} = \frac{k_n}{1 + K} \left( 1 - \frac{M_f}{W} \right) \quad (11)$$

Thus, the gross composition of the animal may be determined from a knowledge of (1) the weight of the animal, (2) the body density of the animal, (3) the densities of fat, muscle, and bone, and (4) the ratios of muscle, skin, and nervous tissue to bone. In what follows, Equations 7 to 11 are established, and their use in the study of human body composition is indicated.

*Fat Content of Eviscerated Guinea Pigs*—On an animal such as the guinea pig it is possible to determine the body composition directly, as well as to calculate it theoretically, thus comparing the two values for a test of the theory.

In Paper I (1), the relationship of  $M_f/W$  to  $G$  in hairless, eviscerated carcasses was determined by direct methods, yielding what is in effect a plot of Equation 7. Even without analysis, the plot furnishes an obvious point of justification (see Fig. 1). Equation 7 predicts that the plot of  $M_f/W$

versus  $G$  should be a rectangular hyperbola<sup>1</sup> displaced from its principal  $x$  axis.

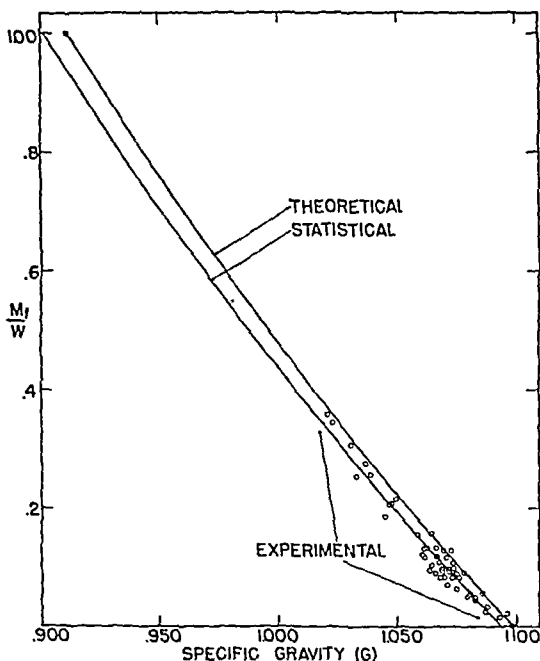


FIG. 1. The abscissa,  $G$ , represents the body specific gravity of the guinea pig, and the ordinate,  $M_f/W$ , represents the ratio of fat to body weight. The equation for the statistical line was obtained as described by Rathbun and Pace (1). The equation for the theoretical line is discussed in the text.

The theoretical curve of  $M_f/W$  also appears on Fig. 1, and will be discussed after consideration of other quantities necessary for the computation.

<sup>1</sup> That the nature of the relationship between the mass of any body component and the average body density is not linear but hyperbolic is deducible from purely dimensional considerations. The per cent of any component has the dimensions  $(M)_c/(W)$ , and the average density has the dimensions  $(W)/(V)$ . If the relationship were linear, the constant of proportionality would have to be  $(M)_c(V)/(W)(W)$ , while if the relationship were hyperbolic, the constant of proportionality would then be  $(M)_c/(V)$ . In the former case, the complexity of the constant and the fact that it contains the dimensions of one of the supposed variables argue against the assumption. The second is therefore favored as the simpler and more reasonable alternative.

## EXPERIMENTAL

*Densities of Tissues*—The density of muscle was determined (3) by matching with standard  $\text{CuSO}_4$  solutions. The mean of determinations on thirty animals was 1.066.

By direct determination, the density of general body fat obtained by extraction was found to be 0.912. That there is no significant difference between the densities of perirenal, intermuscular, and subcutaneous fat can be inferred from the fact that the  $\text{CuSO}_4$  method gives respectively 0.927, 0.921, and 0.921 for these composite tissues.

The average density of the entire skeleton was determined on three guinea pigs. For this purpose all the bones were dissected out and cleaned individually, and the displacement of water by the entire mass measured.

The results agreed well among themselves: 1.430, 1.441, and 1.433 (mean = 1.43), and with the data of Tsai and Lin (4) for non-cleft bone. These authors also give the density of nervous tissue as between 1.04 and 1.05, a fact which made possible Assumption 4.

Direct determination of the density of shaved skin, scraped clean of subcutaneous fat, gives 1.06, and shows that the actual amount of skin is 40.4 per cent of the "skin" (including hair) removed in the usual skinning operation. It is also of interest to note that skin density may be calculated as follows:<sup>2</sup> From the data of Williams (5), and if the density of protein is assumed to be about 1.25 (6), the following composition is obtained: water 66, protein 25, fat 7, inorganic salts, etc., 2 (total 100) g.; approximate volume, 66, 20, 8, and 0 (total 94) cc. respectively. Whence the average density is  $100/94 = 1.06$ . This figure is in close agreement with that obtained by actual measurement and further substantiates Assumption 4.

*Ratios  $k_m$ ,  $k_s$ , and  $k_n$* —The ratio of muscle mass to bone mass,  $k_m$ , was determined in the same three animals referred to above. So far as was possible the entire musculature was dissected out, freed of fat, and collected for weighing. From the data of Table I, a mean value of 5.34 was obtained for  $k_m$ .

A value for  $k_s$  can be determined from the weight of bone and of skin freed of subcutaneous fat and hair by multiplying the "skin" weight by 0.404 as stated above. The mean was found to be 1.95.

$k_n$  defies simple, direct determination; consequently, its value was obtained by indirect means. Judging from a comparison between the diameters of peripheral nerves and the girth of the cord, it is estimated that the peripheral nervous system is about two-thirds the mass of the cord. Thus, one may arrive at an estimate of the mass of the entire nervous

<sup>2</sup> For this calculation, we are indebted to Lieutenant (j.g.) R. E. Eakin, H(S) U. S. N. R.

system. The data of Donaldson (7) give the ratio mass of spinal cord per mass of brain as 0.36; whence it follows that the entire nervous system is about 1.6 times the mass of the brain (for this order). From an allometric determination on the data of Crile and Quiring (8) for the guinea pig, brain weights for our animals (Table II) have been estimated.

TABLE I  
Ratio of Mass of Muscle and Skin to Bone

Guinea pig No.	Weight	Muscle	"Skin"	40.4 per cent "skin"	Bone	$k_m$	$k_s$
	gm.	gm.	gm.		gm.		
1.	572	172.1	150	60.6	32.3	5.47	1.88
2	639	164.6	196	79.2	35.8	4.76	2.21
3	699	222.4	175	70.7	40.0	5.80	1.77

TABLE II  
Ratio of Mass of Central Nervous System to Bone of Guinea Pig

Weight	Brain	Nervous system	Bone	$k_n$
gm.	gm.	gm.	gm.	
572	4.2	6.72	32.3	0.210
639	4.3	6.88	35.8	0.197
699	4.4	7.04	40.0	0.176

# DISCUSSION

*Test of Equations*—There are two ways of combining the results in testing Equation 7. First, a plot of Equation 7 may be compared with a statistical fit to the data (Fig. 1). The parallelism between the two curves in the range 0 to 100 per cent fat is interpreted as a substantiation of the theoretical equations. The consistent error in the direction of yielding low values of  $G$  may well be due to the adherence of air bubbles to the animal's surface and within its ears. A volume of air of about 2 cc. would cause the experimental curve to coincide with the theoretical. At present experiments are in progress to check this question by other specific gravity methods.

Second, Equation 7 may be solved for  $G(0)$ , i.e., the value of  $G$  for  $M_f/W = 0$ , giving,

$$G(0) = \frac{(1 + K)D_m D_b}{D_m + K D_b} \quad (12)$$

Substituting the experimental results in Equation 12, we obtain the following:

$$G(0) = \frac{(1 + 5.34 + 1.95 + 0.2)(1.066)(1.43)}{(1.066) + (5.34 + 1.95 + 0.2)(1.43)} = 1.099$$

an intercept value which is practically coincident with an experimentally measured mean specific gravity value of 1.098 obtained on whole animal aliquot samples rendered fat-free by extraction as described in Paper I (1).

On the grounds of the agreement between theory and experimental data, two useful applications follow.

*Composition of Whole Guinea Pigs*—To this point, both the theoretical application and the experimental data have referred to eviscerated carcasses rather than to the intact animal. For reasons of technical convenience, the data of Paper I (1) were obtained on the eviscerated preparation.

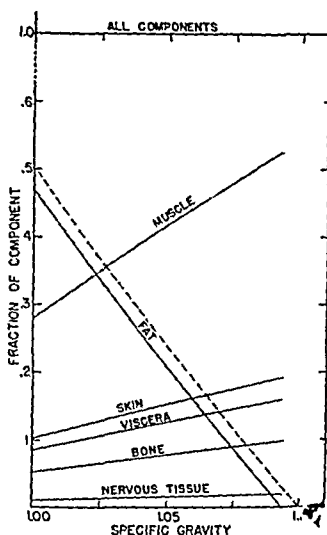


FIG. 2. For any given specific gravity, the tissue fraction of the total body mass may be obtained; thus for example, when  $G = 1.05$ ,  $M_f/W$  is about 0.2, and if the guinea pig weighs 500 gm., it contains  $0.2 \times 500 = 100$  gm. of fat. The dotted line represents the relation of the fat fraction to body specific gravity for man.

However, there is no theoretical obstacle to an extension which includes the viscera. Thus, introducing  $M_v$ ,  $D_v$ , and  $k_v$  as the mass, density, and bone ratio respectively of the viscera, one has on solution a set of equations identical with Equations 7 to 11, except that now one defines

$$K = k_m + k_s + k_n + k_v \quad (13)$$

Extrapolating the plot of visceral fat fraction *versus* visceral density to zero fat fraction gives 1.06 for visceral density. Furthermore, comparison of bone weights (obtained by multiplying the weight of the eviscerated carcass by the factor of Equation 8) with the weights of fat-free viscera (1)

yields a set of values for  $k_*$  whose mean is 1.62. If this  $k_*$  is combined with previous  $k$  values and substituted in Equation 13, it is found that for the hairless intact animal  $K = 9.11$ . When this value of  $K$  is used in Equations 7 to 11, a final result for the body composition of intact, hairless guinea pigs as a function of body density is expressed by the equations  $M_f/W = 5.501 (1/G) - 5.031$ ,  $M_b/W = 0.5965 - 0.5440 (1/G)$ ,  $M_m/W = 3.1840 - 2.9045 (1/G)$ ,  $M_s/W = 1.1640 - 1.0617 (1/G)$ ,  $M_n/W = 0.1193 - 0.1088 (1/G)$ ,  $M_v/W = 0.9663 - 0.8814 (1/G)$ , and is depicted graphically in Fig. 2.

*Application to Man*—As remarked above, the importance and merit of any indirect method of body analysis lie chiefly in its applicability to the living human being. Of the four requisites for the application of the equations it is clear that three can be readily met by the data. The weight and body density of human subjects are accurately determinable quantities (2). In the absence of any facts to the contrary, the *composite* (e.g. muscle with slight amounts of fat) tissue densities for man are the same as those for the common experimental mammals. The technical problem, therefore, reduces to the determination of the  $k$  values. It may be possible to obtain three out of four ( $k_m$ ,  $k_n$ , and  $k_*$ ) directly from roentgenographs;  $k_*$  can be estimated with comparative ease from measurements on cross-sections of human skin and from body surface areas by calculation with empirical formulae (e.g. that of DuBois). The substitution of the various values in Equations 7 to 11 can then be expected to yield gross composition of the human body with reasonable precision. Further work along these lines is in progress. However, even at the present stage of investigation it is possible to deduce a useful provisional equation which gives human fat content as a function of body density. If Equation 7 is written in terms of Equation 12, the following is obtained.

$$\frac{M_f}{W} = \frac{D}{G(0) - 1} \left( \frac{G(0)}{G} - 1 \right) \quad (14)$$

The density of human fat is given as 0.918 in tables such as those of Hodgman (9). In view of the fact that the range of  $G$  for guinea pigs (1) is virtually identical to that for man (2, 10), it may be assumed that  $G(0)$  for man is very close to  $G(0)$  for the guinea pig, i.e. in the neighborhood of 1.10. Substituting for  $D_f$  and  $G(0)$  in Equation 14, we obtain for man  $M_f/W = 5.548 (1/G) - 5.044$ . For purposes of comparison, the graph of this equation is shown in Fig. 2. Calculation of other components awaits further experimental data.

It is a pleasure to acknowledge the generous collaboration and counsel of Lieutenant I. Gersh, H(S), U. S. N. R., and the Pathology Facility of the Naval Medical Research Institute.



## SUMMARY

By treating the body as a five phase system, equations are developed which give the amount of each tissue component as a function of body weight and average body density.

These equations are based on the assumption that there is a lean body mass in the guinea pig and in man of relatively uniform composition. Fat is regarded as the only component that exhibits appreciable relative variation. The quantitative data obtained on these guinea pigs substantiate this assumption.

In man, values for specific gravity comparable to those of the guinea pig have been obtained. On the basis of available data, therefore, the relationship between fat and body density as determined for the guinea pig appears to be directly applicable to man.

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## STUDIES ON BODY COMPOSITION

### III. THE BODY WATER AND CHEMICALLY COMBINED NITROGEN CONTENT IN RELATION TO FAT CONTENT\*

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The resolution of the animal body into its components, both chemical and morphological, is generally regarded as only approximate because of individual variation. However, the over-all uniformity of mammalian tissues with respect to chemical composition is filled with implication and has led to such diverse and well known generalizations as Claude Bernard's concept of a constant *milieu intérieur*, Macallum's theory of the universal origin of mammalian forms in the oceans, and the concept of the "active protoplasmic mass" of Talbot and Benedict.

Perhaps the only whole body component, and certainly by far the chief one, which may fluctuate widely in the so called "normal" population is fat. To be sure, considerable variability occurs with many other substances; however, these variations are inconsequential when compared with that of fat on a gravimetric basis. It has been shown in Paper I in this series (1) that the whole body fat of guinea pigs selected from laboratory stock animals may vary from 1.5 per cent to 35.8 per cent of the body weight.

In the following, data are presented which indicate the relative constancy of total body water and nitrogen when expressed on a fat-free basis, together with some of the implications of this correction.

#### EXPERIMENTAL

In the series of 50 normal guinea pigs examined for body specific gravity and fat content as described in Paper I (1), determinations of total body nitrogen and body water were made. Water content was obtained as the difference between wet weight and dry weight, the latter being measured as described previously (1). Nitrogen analyses were obtained by the macro-Kjeldahl method on aliquot samples of the pulverized whole animal, which included skin and bone but which did not include hair or viscera for the reasons given (1). The data for this series of animals are summarized in Table I.

\* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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TABLE I

*Original Data on 50 Eviscerated Guinea Pigs Taken from Laboratory Stock\**

Guinea pig No.	Total water	Total nitrogen	Guinea pig No.	Total water	Total nitrogen
	gm.	gm.		gm.	gm.
79	206.5	10.1	137	414.2	19.3
87	319.0	14.9	138	155.2	7.9
88	333.5	15.6	139	376.1	18.3
89	384.6	17.9	140	282.6	13.6
90	415.9	18.8	141	325.2	14.9
91	392.8	15.9	142	244.2	10.1
92	288.4	13.3	143	150.1	8.2
93	369.5	16.1	144	314.0	13.8
99	405.3	20.0	150	329.5	15.9
100	387.5	18.3	151	205.0	9.2
101	451.7	20.5	152	244.4	10.9
102	392.0	20.2	153	299.3	14.5
105	281.2	13.3	154	413.7	18.8
106	217.2	10.1	155	355.1	17.0
107	295.9	13.5	156	326.8	15.4
108	233.6	11.1	157	313.2	16.5
109	255.7	12.6	158	198.2	8.3
114	260.9	16.3	159	302.5	12.9
115	161.1	10.8	160	231.5	10.3
116	198.7	10.8	161	243.1	10.8
117	254.5	13.3	162	358.4	16.4
118	218.5	13.0	163	335.0	15.4
119	190.7	11.3	164	375.5	18.4
120	179.1	10.6	165	356.4	16.8
136	294.7	13.3	166	327.0	18.7

\* The eviscerated wet weights and gm. of total fat for these animals are given in Paper I of this series (1).

TABLE II

*Comparison of Mean Water Content and Its Standard Deviation of Various Tissues from Twenty Guinea Pigs on Wet Weight Basis and on Fat-Free Wet Weight Basis*

Tissue	Fat, per cent wet weight		Water, per cent wet weight		Water, per cent fat-free wet weight	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Perirenal fat	84.13	4.79	10.25	5.97	81.78	3.45
Muscle..	2.36	7.83	74.65	0.66	76.48	0.44
Liver...	3.48	1.32	69.06	1.77	71.55	1.46
Adrenal	14.88	3.52	65.80	2.42	77.32	0.83
Nerve .	14.65	2.57	62.66	2.51	73.40	1.21
Tendon...	6.96	2.11	55.68	2.20	59.84	1.86

In addition to the determinations on whole animals, the water content and fat content of various tissues from the guinea pig were obtained. Water content was again measured by difference between wet and dry weights, and fat content was measured by the fiberglas bag technique (1). These data are summarized in Table II. The various tissues represented were obtained in connection with another study (2) and were skeletal muscle from the thigh, liver, adrenal tissue, tendon, nerve, and perirenal fat. In each case the mean water content and standard deviation were calculated for both the original tissue and fat-free tissue.

A similar calculation was made for the nitrogen and water contents of the whole animal, and these computations are summarized in Table III.

TABLE III

*Comparison of Whole Body Water and Chemically Combined Nitrogen Content of 50 Guinea Pigs on Various Bases*

	Mean	Standard deviation	Coefficient of variation
Water, % . . .	63.50	5.56	8.8
" in fat-free tissue, %	72.42	2.11	2.9
Nitrogen, % dry weight	8.65	1.87	21.6
" % wet "	3.08	0.43	14.0
" % fat-free dry tissue	12.72	0.78	6.1
" % " wet "	3.52	0.27	7.7

## DISCUSSION

It is evident from the data presented in Table II that the water content of various tissues of the body is more nearly constant when expressed on a fat-free basis. This is in full accord with the views of Hastings (3). It is also of interest that the mean water contents of various tissues differ among themselves even when expressed on this basis. However, these means occupy a considerably narrower range than when expressed on a basis uncorrected for fat. In particular it is of significance that a tissue as high in fat content as perirenal fat tissue exhibits a water content comparable to that of other tissues when this correction is made.

Table III contains a summary of the analyses of the whole animal. It may be seen that both water content and nitrogen content are remarkably constant when expressed on a fat-free basis. This fact is strikingly shown in Fig. 1 in which the actual gm. of water and nitrogen are plotted against the fat-free body weight.

Ashworth and Cowgill (4) have presented data on the body composition of rats and included values for total fat, water, and nitrogen. For purposes of comparison, their data were treated in the same fashion and are also plotted in Fig. 1.

It may be seen that both water and nitrogen exhibit straight line relationships with fat-free body weight, and yield high correlation coefficients

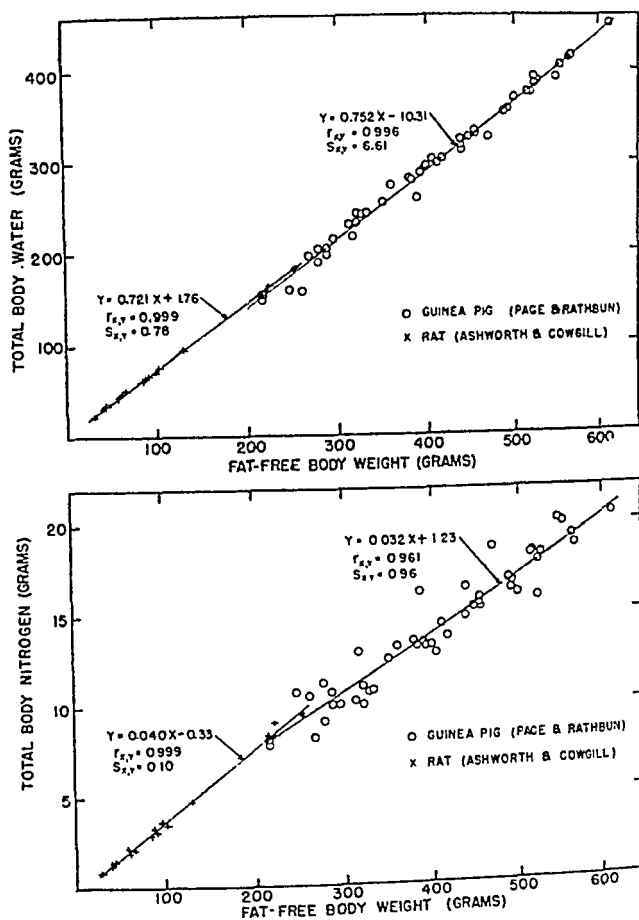


FIG. 1. The upper graph represents the total body water plotted against the fat-free body weight for each animal in our series of guinea pigs and for each animal in the series of rats studied by Ashworth and Cowgill (4). The lower graph represents the total body nitrogen plotted on the same basis. The equations were obtained by the method of least squares.

when the data are fitted by the method of least squares. On the other hand, when the total gm. of body water or nitrogen are plotted against the actual body weight, the relationship appears curvilinear. An explanation for the

latter may be found in the fact that older animals, and hence animals of larger body weight, tend to contain a greater percentage of fat than the younger and smaller animals. Considering fat as a diluent of the lean body mass, the nitrogen and water percentages of the whole body reflect changes in the fat content because of the relative constancy of water and nitrogen in the lean body mass.

From the foregoing it becomes obvious that the practice of expressing experimental data in terms of per cent of the total dry weight should be rather an expression of data in terms of per cent of the total water present, which would be independent of the fat content. Such a reference standard

TABLE IV  
Summary of Mean Whole Body Water and Chemically Combined Nitrogen Content for Adults of Various Mammals

Species	Bibliographical reference No.	Per cent water	Per cent nitrogen	Per cent fat	Per cent water in lean mass	Per cent nitrogen in lean mass
Rat.....	(5)	65.3	3.54	9.0	71.8	3.89
".....	(4)	63.6	3.04	14.6	74.4	3.57
".....	(6)	61.5	3.01	15.3	72.6	3.56
Guinea pig.....	(5)	67.1	3.18	10.0	74.2	3.51
" ".....	Our data	63.5	3.08	12.3	72.4	3.52
Rabbit.....	(5)	69.2	2.91	7.8	73.5	3.09
".....	(7)	74.3		2.5	76.3	
Cat.....	(5)	66.7	3.22	7.9	72.4	3.50
Dog..	(7)	59.5		20.1	74.5	
".....	(8)	59.1		15.4	69.9	
Monkey.....	(7)	68.5		6.5	73.3	
Mean..					73.2	3.52

is then equivalent to the expression of data in terms of per cent of the total nitrogen and, judging from Fig. 1, would appear to be better.

Hatai (5) in a classical work on the body composition of the rat at various ages gives mean analytical values for total fat, water, and nitrogen. He has also gathered data on various other species of mammals. Recalculation of his data, together with that of others, so that the nitrogen and water content of the whole body is expressed on a fat-free basis, accounts for some of the discrepancies noted by him.

His demonstration of the large decrease in water content during early growth remains unaffected by the fat correction. However, the water content of adults for the various species given becomes quite constant when expressed on the fat-free basis. A summary of such data from several sources (4-8) is given in Table IV. When the wide variety of

methods used to obtain the data is considered, and also the fact that the data given by Hatai have been corrected for fat on the basis of mean percentages rather than for each individual, the agreement among species is surprisingly good, and it is not too far fetched to apply the mean value of water in the lean body mass of 73.2 per cent to man.

The concept of a lean body mass which is comparatively constant in composition in man (8) and among the adults of various species of mammals receives considerable substantiation from the available experimental evidence. Thus, the assumptions made in Paper II (9) in establishing the theoretical relationships of the various tissue components of the body appear to be justified experimentally thus far.

It was shown (1) that the female guinea pigs contained more fat than male guinea pigs of a corresponding fat-free body weight. When the water and nitrogen contents of the males and females are compared on this basis, however, there is no difference. It is interesting to speculate on the question of how many of the quantitative differences in function between the male and female would be equalized if expressed on the basis of the lean body mass.

In view of the relative constancy of the water content of the lean body mass, it is possible to derive a simple equation describing the relationship between water content and fat content of the whole animal mass thus:

$$\% \text{ fat} = 100 - \frac{\% \text{ water}}{0.732} \quad (1)$$

In Paper I of this series (1) an equation describing the relationship of human whole body specific gravity and fat content was presented in the form

$$\% \text{ fat} = 100 \left( \frac{5.548}{\text{sp. gr.}} - 5.044 \right) \quad (2)$$

By substitution in Equation 1

$$\% \text{ water} = 100 \left( 4.424 - \frac{4.061}{\text{sp. gr.}} \right) \quad (3)$$

It is therefore possible to predict from the whole body specific gravity both the fat content and water content with a reasonable degree of accuracy. A generalization of this kind is of course subject to considerable limitation; *e.g.*, in the case of very young animals, in the case of marked variation in skeletal proportion, and in the case of disturbed water balance. However, with normal adult animals it can serve as a useful approximation, and Equation 3 may be applied to man, together with Equation 2. As a check on this assumption it is possible to make the calculation suggested by Behnke (8) on the theoretical total amount of gaseous nitrogen dissolved.

in the body of a man of whom the body specific gravity is known, and then to compare the calculated values with the experimentally determined quantity of dissolved nitrogen gas. Good agreement is obtained between the experimental value and the calculated value.

In the field of energy metabolism considerable data have been obtained on the metabolic rates of a great variety of living forms; yet difficulty has been encountered in making generalizations, owing to the lack of a universally accepted standard of reference. Kleiber (10) has emphasized this in a recent review on energy metabolism. Ashworth and Cowgill (4) have been able to show that fat-free dry weight is a better standard of reference for basal metabolic rate in rats than is dry weight or live body weight. Further work along these lines is indicated.

#### SUMMARY

1. A study of the water content and chemically combined nitrogen content of 50 guinea pigs reveals that the proportion of these substances to body weight is constant, provided that the body fat is considered.

2. Water was found to constitute 72.4 per cent of the fat-free body mass, and exhibited a standard deviation of 2.11 per cent. The range of the original live weights represented was from 304 to 1000 gm.

3. Chemically combined nitrogen constituted 3.52 per cent of the lean body mass, with a standard deviation of 0.27 per cent.

4. These values agree closely with those calculated for other mammals on a fat-free basis. No sex differences were found in water and nitrogen content.

5. It is believed that the concept of a lean body mass that is relatively constant in gross chemical composition, in which body fat may be considered to act as a diluent, is supported by the available experimental evidence. Some of the implications of this concept are discussed.

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# THE ELECTROPHORETIC ANALYSIS OF STORED LIQUID HUMAN PLASMAS

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Human plasma, prepared under sterile conditions by closed vacuum technique and containing 5 per cent of glucose and 0.01 per cent of merthiolate, may be stored at room temperature in the liquid state for at least 1 year and still be used for transfusion "with safety and benefit" (1). The coagulation factors and the complement are lost (2), but the incidence of untoward reactions is significantly lower than with fresh plasma (1).

Physicochemical studies on liquid plasmas stored for 1 year (3) and 2 years (4) have given evidence of changes in the electrophoretic pattern. Four samples of pooled human plasma which had been prepared according to the above technique and stored at room temperature for 3 years were supplied by Lieutenant Commander Eugene L. Lozner of the Naval Medical Research Institute, Bethesda, Maryland, so that the changes in the electrophoretic pattern produced by the longer storage could be determined. As controls he supplied two samples of fresh pooled human plasma: one, designated Plasma A, was prepared by closed vacuum technique but contained no added glucose or merthiolate, and the other, designated Plasma B, was prepared exactly as the stored plasmas.

## EXPERIMENTAL

Electrophoresis of the plasmas was carried out by the Tiselius method (5) against two different buffer solutions, one of pH 7.9 containing 0.15 M NaCl and 0.02 M sodium phosphates, and one of the same pH and electrolyte composition containing in addition 5 per cent of glucose; the required volume of each plasma was diluted with an equal volume of the buffer solution to be used, and dialyzed against four changes of the same buffer solution. Photographs were taken at regular intervals by both the Longsworth (6) and the Svensson (7) methods. A current of 40 milliamperes, corresponding to a potential gradient of 6.0 volts per cm., was used for the glucose-free solutions. In the glucose-containing solutions the electrical resistance was higher than in the glucose-free solutions, so that a current of 40 milliamperes corresponded to a potential gradient of 6.9 volts per cm. and produced convection effects; a current of 27 milliamperes, corresponding to a potential gradient of 4.6 volts per cm., was therefore used for these solutions. In the glucose-containing solutions the mobilities

were reduced on account of the increased viscosity, or *mechanical* resistance, but, because of the higher potential gradient per unit of current resulting

TABLE I

*Electrophoretic Mobilities of Stored Human Plasmas*

Buffer, pH 8, 0.15 M NaCl, 0.02 M phosphates.  $T = 0^\circ$ .

Electrophoretic mobility =  $u = \text{sq. cm. per volt second.}$

Plasma No.	Arm of U-tube*	A	$\alpha_1$	$\alpha_2$	$\alpha_3$	$\beta$	F	$F_2$	$\gamma_2$	$\gamma$	$\delta$ or $\epsilon$	Remarks
290-4	D.	5.0	3.3		2.0							Stored 3 yrs.; run in 5% glucose
	A.	4.9	3.8		2.2						0.3	
291-4	D.	5.0	3.4		2.4						0.8	" 3 " " " 5% "
	A.	5.1	3.8		2.4						0.2	
292-4	D.	4.6	3.2		2.2						0.5	" 3 " " " 5% "
	A.	4.7	3.7		2.4						0.4	
197-5	D.	5.2	3.6		2.7						0.6	" 3 " " " 5% "
	A.	5.2	3.8		2.7						0.3	
B	D.	4.1	2.6		2.1	1.6				1.0		Fresh plasma stored with 5% glucose; run in 5% glucose
	A.	4.1	3.1		2.3	1.8				1.0	0.2	
A	D.	4.1	2.7		2.3	1.8	1.6			1.2		Fresh plasma stored without glucose; run in 5% glucose
	A.	4.1	3.0		2.5	2.0				1.0	0.3	
290-4	D.	6.0	3.9		2.4						0.5	Stored 3 yrs.; dialyzed glucose-free
	A.	6.1	4.2		3.0						0.1	
291-4	D.	5.7	3.9		2.4						0.8	" "
	A.	5.7	4.2		2.7						0.3	
292-4	D.	6.1	4.0		2.8						0.7	" "
	A.	6.1	4.6		2.7						0.3	
197-5	D.	6.2	4.1		2.8						0.7	" "
	A.	6.2	4.7		2.9						0.3	
B	D.	5.0	4.3	3.2	2.6	1.9			1.4	1.1		Fresh; stored with 5% glucose; dialyzed glucose-free
	A.	5.1	4.3	3.7	2.9	2.2				1.3	0.1	
A	D.	4.8	4.0	3.2	2.5	1.8				1.0		Fresh; stored without 5% glucose
	A.	4.9	4.2	3.7	2.8	2.1				1.1	0.2	
"	D.	5.0	4.0	3.1	2.7	2.0	1.7			1.3		" 5% glucose added, then dialyzed away
	A.	5.0	4.2	3.6	2.8	2.2				1.0	0.2	
"	D.	4.7		3.3	2.9	2.6	2.2			1.2		Heated 10 min. at 65° (no glucose present)
	A.	4.9		3.7	3.5	2.8	2.0			1.0	0.2	
B	D.	5.0		3.3	3.2	2.5			1.7	1.4		Heated 10 min. at 65°, then dialyzed glucose-free
	A.	5.1	4.0	3.8	3.5	3.3				1.7	0.7	

All mobilities have been multiplied by  $10^5$ .

\* A. represents ascending; D., descending.

from the increased *electrical* resistance, the distances migrated during the passage of equal quantities of electricity were practically the same as in the glucose-free solutions.

Control human Plasma A was examined under three conditions: (a)

TABLE II

*Per Cent Composition of Stored and Fresh Human Plasmas*

Computed from areas of electrophoretic diagrams in buffer of pH 8, containing 0.15 M NaCl and 0.02 M sodium phosphates.

Plasma No.	Arm of U-tube*	A	$\alpha_1$	$\alpha_2$	$\beta$	F	$F_2$	$\gamma_2$	$\gamma$	$\Delta n \times 10^4$	Remarks
290-4	D.	65.5		29.3	5.2					561	Stored 3 yrs.; run in 5% glucose
	A.	63.9		30.1	6.0						
291-4	D.	66.7		27.0	6.3					643	" "
	A.	66.7		27.0	6.3						
292-4	D.	65.9		29.1	5.0					603	" "
	A.	69.3		26.7	4.0						
197-5	D.	70.2		25.4	4.4					535	" "
	A.	69.0		25.7	5.3						
B	D.	60.0		12.0	7.6	6.6			13.8	649	Fresh plasma stored with 5% glucose; run in 5% glucose
	A.	56.4	0.8	10.5	7.3	5.8			19.2		
A	D.	58.9	2.1	13.1	6.4	4.7	2.3		12.5	570	Fresh plasma stored without glucose; run in 5% glucose
	A.	57.8	1.0	12.0	5.5	6.1			17.6		
290-4	D.	64.4		31.0	4.6					640	Stored 3 yrs.; dialyzed glucose-free
	A.	65.8		27.3	6.9						
291-4	D.	63.6		32.1	4.3					602	" "
	A.	66.8		28.0	5.2						
292-4	D.	63.9		31.0	5.1					640	" "
	A.	66.5		28.7	4.8						
197-5	D.	67.1		27.1	5.8					608	" "
	A.	68.6		26.4	5.0						
B	D.	58.6	2.6	11.5	8.0	5.9		4.0	9.4	668	Fresh; stored with 5% glucose; dialyzed glucose-free
	A.	60.1	1.7	10.5	7.3	5.7			14.7		
A	D.	56.7	3.1	9.8	10.6	5.4			14.4	631	Fresh; stored without 5% glucose
	A.	59.4	1.7	9.9	9.9	5.7			13.4		
"	D.	57.7	2.6	13.3	6.1	5.8	1.7		12.8	617	Fresh; 5% glucose added, then dialyzed away
	A.	59.8	1.3	12.5	5.8	6.6			14.0		
"	D.	35.8		53.7		1.7			8.8	562	Heated 10 min. at 65° (no glucose present)
	A.	38.3		50.9	3.5	1.6			5.7		
B	D.	44.7		42.9				4.0	8.4	612	Heated 10 min. at 65°, then dialyzed glucose-free
	A.	46.5		33.3	6.4				13.8		

Values for the ascending arm have been calculated on the assumption that the percentage dilution at the  $\delta$ -boundary is the same for all constituents.

\* A. represents ascending; D., descending.

untreated (*i.e.*, no added glucose) after standing 6 days at room temperature; (b) after standing 7 days longer at room temperature in the presence of 5 per cent glucose; (c) same as (b), but dialyzed glucose-free. The elec-

trophoretic patterns are shown in Fig. 1. Control Plasma B, to which glucose was added to a concentration of 5 per cent at the time of preparation, stood 23 days at room temperature before samples were withdrawn for electrophoresis; it was examined both in the presence of 5 per cent glucose and after removal of the glucose by dialysis (Fig. 2). The four plasmas which had been stored for 3 years at room temperature were also examined both in the presence of 5 per cent glucose and after removal of the glucose by dialysis (Fig. 3). The mobilities are listed in Table I, and the percentage composition of each plasma is given in Table II.

In view of the possibility that the changes produced by prolonged storage in the liquid state at room temperature might result from spontaneous reactions progressing slowly at room temperature but accelerated at higher temperatures, samples of control Plasmas A and B were subjected to heat treatment. 8 ml. of each plasma were placed in a 25 ml. Erlenmeyer flask and heated for 10 minutes, with constant swirling, in a water bath maintained at 65°; the samples were chilled immediately afterwards. Flocculation occurred in both plasmas, but to a greater extent in Plasma A, which contained no added glucose and was therefore undiluted at the time of heat treatment, than in Plasma B, which when collected had been diluted with 50 per cent glucose solution to a final glucose concentration of 5 per cent. Both plasmas (centrifuged clear) were examined by electrophoresis (Figs. 1 and 2) after dilution with, and dialysis against, the glucose-free buffer.

#### DISCUSSION

*Effect of Added Glucose on Electrophoretic Pattern of Fresh Human Plasma*—The electrophoretic pattern for the untreated Plasma A (Fig. 1) in the glucose-free buffer was similar to that previously found for normal human plasma in this buffer solution ((8) Fig. 1), except that the  $\alpha_2$ -globulin was present in somewhat higher concentration, and the  $\beta$ - and  $\gamma$ -globulins were present in somewhat lower concentrations ((9) Table 2).

The presence of 5 per cent glucose produced several changes in the pattern. The  $\alpha_1$ -globulin was no longer a separate peak, but only a slight plateau between the albumin and  $\alpha_2$ -boundaries. The concentration of the  $\alpha_2$ -globulin was increased at the expense of the  $\beta$ -globulin, and there was a tendency, more pronounced in the descending arm of the U-tube than in the ascending arm, for fusion of these two boundaries. The descending fibrinogen boundary was distorted and broken into two parts.<sup>1</sup>

<sup>1</sup> In Fig. 1, the  $F_x$  boundary of Plasma A in 5 per cent glucose appears, because of incomplete resolution, to be part of the  $\gamma$ -globulin boundary. The association with the fibrinogen boundary (as in Plasma A, dialyzed glucose-free, Fig. 1) could be seen more clearly in later diagrams for glucose-containing Plasma A, and was shown also by the percentage composition (Table II).

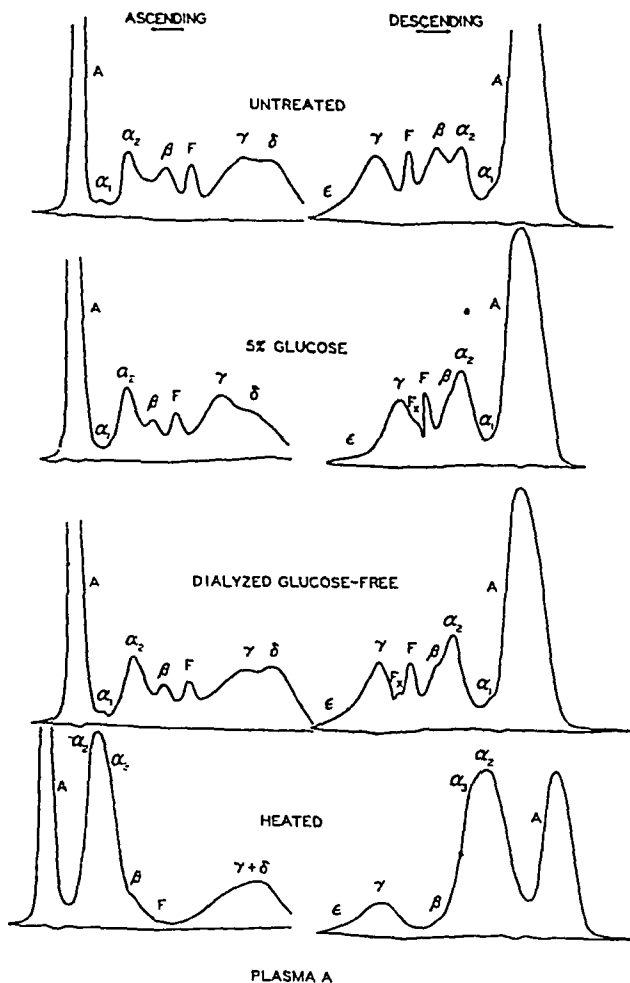


FIG. 1. Electrophoretic patterns of Plasma A (tracings of Svensson diagrams). pH 7.9, 0.15 M NaCl, 0.02 M sodium phosphates. The plasma stood 6 days at room temperature before samples were removed for electrophoresis. Glucose-treated samples stood 7 days longer at room temperature in contact with glucose. Untreated, 442 coulombs (approximately 3 hours at 40 milliamperes); 5 per cent glucose, 435 coulombs (approximately 4.5 hours at 26.7 milliamperes); dialyzed glucose-free, 468 coulombs (approximately 3 hours at 40 milliamperes); heated 10 minutes at 65°, 493 coulombs (approximately 3.5 hours at 39 milliamperes).

Between the fibrinogen and  $\gamma$ -globulin boundaries a precipitate appeared which was scarcely perceptible during the first 5 hours of the experiment but which became opaque overnight.

After the removal of the glucose, the  $\alpha_1$ -globulin boundary reappeared as a separate peak. The relative concentrations of the  $\alpha_2$ - and  $\beta$ -globulins remained the same as before the removal of the glucose, and the tendency for fusion of the two boundaries in the descending arm persisted. The distortion of the descending fibrinogen boundary and the resolution into two parts persisted also, but the precipitate between the  $\gamma$ -globulin boundary and the fibrinogen boundary caused complete opacity within 2.5 hours and on standing overnight sedimented to the bottom of the cell, leaving only a slight opalescence near the original site.

The electrophoretic pattern for Plasma B, containing 5 per cent of glucose (Fig. 2), resembled in many respects that for Plasma A in 5 per cent glucose. The  $\alpha_1$ -globulin was visible only as a plateau, and the concentrations of  $\alpha_2$ -globulin and  $\beta$ -globulin were very nearly the same as in Plasma A (Table II). The tendency for fusion of the  $\alpha_2$ - and  $\beta$ -boundaries was, however, less pronounced than in Plasma A, the descending fibrinogen boundary was free from distortion, and no precipitation occurred in the descending fibrinogen or  $\gamma$ -globulin boundaries.

The changes produced by removal of the glucose from Plasma B were about the same as for Plasma A. The  $\alpha_1$ -boundary reappeared, and the concentrations of the  $\alpha_2$ - and  $\beta$ -globulins remained the same as before removal of the glucose. Resolution of the descending  $\alpha_2$ - and  $\beta$ -boundaries was improved, however, and although the descending fibrinogen boundary remained free from distortion the descending  $\gamma$ -globulin boundary was partially resolved into two parts. No precipitation occurred in the descending arm.

The changes brought about by the addition of glucose emphasize the fact that each electrophoretic constituent represents not a single protein but a "population of proteins" (10), and that the mobilities of many are sensitive to changes in environment. The persistence of some of the changes after removal of the glucose by dialysis suggests that the glucose may either effect certain permanent changes, or accelerate spontaneous changes which proceed more slowly in the absence of glucose. The greater sharpness and symmetry of the boundaries of Plasma B, as compared with Plasma A under corresponding circumstances, and the absence of flocculation during electrophoresis of Plasma B appear, in view of the greater age of Plasma B, to indicate a considerable protective action by the added glucose. A possible further indication of this protective action may be found in the behavior of Plasma A before and after removal of the 5 per cent glucose. The precipitate which formed at the fibrinogen boundary in the descending arm during electrophoresis flocculated less rapidly in the presence of 5 per cent glucose than after removal of the glucose by dialysis.

*Electrophoretic Behavior of Stored Human Plasma and Comparison with*

*Heated Control Plasma*—The four plasmas which had been stored in the liquid state for 3 years at room temperature showed remarkable uniformity in their electrophoretic patterns (Fig. 3), with respect to both the mobilities of the fractions (Table I) and the percentage composition (Table II).

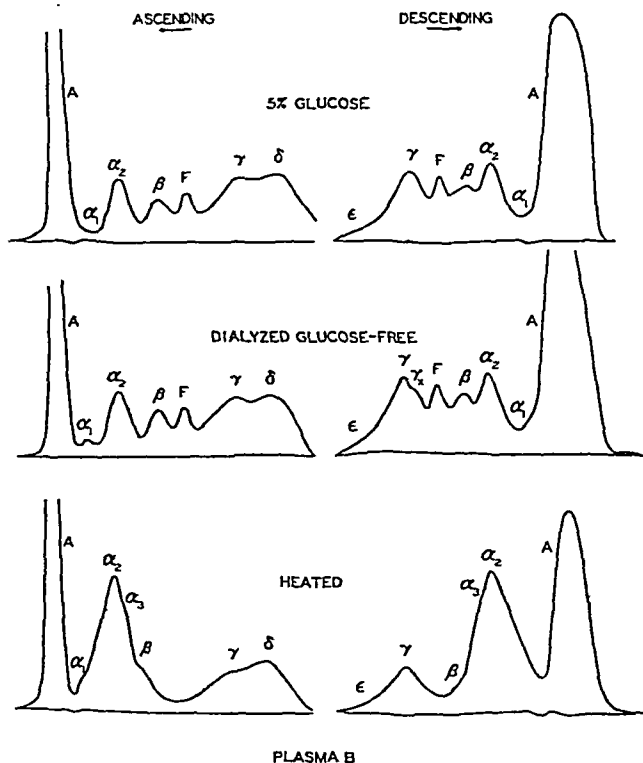


FIG. 2. Electrophoretic patterns of Plasma B (tracings of Svensson diagrams). pH 7.9, 0.15 M NaCl, 0.02 M sodium phosphates. The plasma stood 23 days at room temperature before samples were withdrawn for electrophoresis. 5 per cent glucose, 523 coulombs (approximately 2.5 hours at 39.5 milliamperes plus 1.66 hours at 27.1 milliamperes); dialyzed glucose-free, 433 coulombs (approximately 3 hours at 40 milliamperes); heated 10 minutes at 65°, 494 coulombs (approximately 3.5 hours at 39 milliamperes).

The presence or absence of glucose produced no significant change either in the contour of the patterns or in the percentage composition; the effects of the increased viscosity and electrical resistance caused by the presence of 5 per cent glucose compensated one another, as in fresh plasma, and yielded almost identical patterns for the passage of equal quantities of electricity.



treatment, and postulated a similar mechanism: an unfolding and splitting of the molecules, followed by aggregation. By irradiation of the albumin-rich and globulin-rich fractions of human serum, they found that the mobility of the aggregation product varies with the composition of the mixture subjected to irradiation. In bovine plasma the C component is associated with the  $\alpha$ -globulin boundary (14) as in human plasma; the formation of C component in bovine plasma can be prevented by saturation with glucose previous to heat treatment. In view of the association of C component with the  $\alpha$ -globulin fraction in heated human plasma, it may be of interest to recall that febrile infections have been found to increase the  $\alpha$ -globulin content of human plasma (15-17).

More extensive changes were produced in Plasma A than in Plasma B in consequence of heat treatment. This may have been the result either of the higher protein concentration of Plasma A, which would have facilitated aggregation and thus promoted the formation of C component from the heat-modified molecules, or of the presence of 5 per cent glucose in Plasma B, which, in view of the effects of saturation with glucose on bovine plasma, might be expected to repress somewhat the formation of C component. No further experiments were carried out to determine which was the more important factor.

The augmentation of the  $\alpha$ -globulin by prolonged storage at room temperature, though at the expense of different plasma constituents, suggests that the same type of mechanism may be involved as in heat treatment. The divergences may result both from the varying susceptibilities of the plasma constituents to the primary change (spontaneous denaturation in the one instance, heat modification in the other), and from the more gradual accumulation of the modified molecules, and the greater opportunity for completion of the secondary reaction (aggregation of the modified molecules, or of their fission products (13)) during prolonged storage than during heat treatment.

The results do not, however, permit of explanation by the hypothesis (13) that the electrical homogeneity of the denaturation product is largely due to a "chemically homogenizing process;" i.e., the aggregation of the fission products of the molecules in the proportions in which they are present. The augmentation of the  $\alpha$ -globulin on prolonged storage of the plasma occurred at the expense of the more slowly migrating constituents exclusively; there was no diminution of the more rapidly migrating albumin. It should be pointed out, too, that the denaturation product (C component) of human plasma, after either heat treatment or prolonged storage, was much less homogeneous than that of horse serum after heat treatment (11, 12), or ultraviolet irradiation (13), that of bovine plasma after heat treatment (14), or those of the albumin-rich or globulin-rich fractions of human serum after ultraviolet irradiation (13).

It is difficult to compare these results on 3 year-old plasma with those obtained by electrophoresis of 2 year-old plasma<sup>2</sup> because of the difference in buffer solutions (cf. (9) Table 2). The electrophoretic diagram for 2 year-old plasma, which was available to us through the courtesy of Lieutenant Commander Lozner, appeared to indicate complete loss of  $\gamma$ -globulin and fibrinogen and partial loss of  $\beta$ -globulin, as in 3 year-old plasma; it showed no apparent increase of the  $\alpha$ -globulin, however, and the material lost from the slow boundaries was stated by the investigators (4) to be associated with the albumin fraction.

The increased mobility and increased boundary spreading of the plasma constituents after storage for 3 years had no counterpart in the patterns of the heated plasma, and may have resulted from the action of enzymes present in the plasma. An enzyme resembling trypsin has been prepared from fresh chloroform-treated plasma, and evidence for proteolytic action during prolonged storage of liquid plasma has been found in the increase of non-protein nitrogen (4). The limited degree to which non-protein nitrogen was liberated (equivalent to hydrolysis of less than 2 per cent of the original protein), and the comprehensiveness with which increased mobility was shown by the proteins of the plasma, suggest that if the two effects are manifestations of the same change the non-protein nitrogen must be produced by partial fission of most, or even all, of the molecules, rather than by complete fission of a few.

#### SUMMARY

Pooled human plasma, prepared under sterile conditions by the closed vacuum technique and containing 5 per cent of glucose and 0.01 per cent of merthiolate, was examined by electrophoresis in the Tiselius apparatus after storage in the liquid state at room temperature for 3 years. There was a large increase of  $\alpha$ -globulin at the expense of all the  $\gamma$ -globulin, all the fibrinogen, and part of the  $\beta$ -globulin. The mobilities were 20 to 25 per cent greater than in fresh plasma, and the boundaries were broadened.

Heat treatment of fresh human plasma also caused an increase of the  $\alpha$ -globulin, but at the expense of different plasma constituents, and without increase of the mobilities or broadening of the boundaries.

The addition of 5 per cent of glucose to fresh human plasma produced an increase of the  $\alpha_2$ -globulin at the expense of the  $\beta$ -globulin, with partial fusion of the  $\alpha_2$ - and  $\beta$ -globulin boundaries in the negative arm; it also caused the disappearance of the  $\alpha_1$ -globulin as a separate boundary. The former change persisted after removal of the glucose by dialysis; the latter did not. The electrophoretic patterns of plasmas stored at room temperature for periods of less than a month, with and without the addition of glucose, indicated protective action by the added glucose.

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# THE ESTIMATION OF BASIC ORGANIC COMPOUNDS AND A TECHNIQUE FOR THE APPRAISAL OF SPECIFICITY\*

## APPLICATION TO THE CINCHONA ALKALOIDS

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The chemotherapeutic action of an agent in the intact animal is usually conditioned by two factors (1). The first is the manner and extent to which the substance is capable of participating in the activities of a discrete biological system and thereby producing a particular chemotherapeutic effect. The second, which relates to the over-all physiological disposition of the agent by the animal, is related to the operation of such diverse processes as absorption, distribution, degradation, and excretion. Generally speaking, these processes are integrated in such a fashion that the concentration of the agent in the plasma on any dosage schedule is a reflection of their combined operation, since it is through this medium that most exchanges of the drug occur. Consequently in a definitive appraisal of a drug information of its plasma concentrations and the time these concentrations must be maintained in order to produce a given chemotherapeutic effect will usually be required. Furthermore, the maximal benefit to be derived from a drug in routine therapy is generally difficult to obtain in the absence of these quite different types of information (2).

The collection of such information in the study of drugs of unknown character, to be used for any given purpose, would be considerably facilitated by the availability of reasonably simple analytical procedures. Also, the development of these would be aided were it possible to utilize a scheme for the analysis of organic compounds based upon a series of general reactions. However, the use of a non-selective reaction requires that means be available to examine the specificity of the over-all measurement when applied to the estimation of a particular compound. Such an examination has an advantage if in addition to giving an appraisal of specificity it also yields information on the characteristics of the interfering substances. The latter type of information may then be applied so as to permit a modification of the fundamental procedure in order to introduce the desired specificity.

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A simple general method is described below which, with minor modifications, has been used in the development of methods for the estimation of a number of alkaloids and synthetic basic organic compounds. The principle of the method is that many organic bases combine with certain sulfonic acids to form molecular complexes which are highly soluble in organic solvents, and that the concentration of base in the organic solvent may be determined indirectly through a measurement of the concentration of the sulfonic acid in the organic solvent. The technique used to appraise the specificity of the procedure involves a comparison of the solubility characteristics of the pure compound with those of the substance or substances isolated from the biological material and measured in the analytical procedure.

The development of a procedure for the estimation of cinchonidine in biological fluids is given to illustrate the general method of analysis and the technique involved in assaying and improving specificity. The method is equally applicable to the other cinchona alkaloids.

#### *Procedure for Cinchonidine*

The sulfonic acid chosen for use in the present procedure is methyl orange, since it approaches the ideal acid for the present purpose. It is a water-soluble dye with a high color index; it forms a cinchonidine-methyl orange complex highly soluble in ethylene dichloride and chloroform; it only enters the organic phase in amounts equivalent to the contained base.

Cinchonidine is separated from the biological sample by extraction into ethylene dichloride at an alkaline pH. The ethylene dichloride is washed with alkali to remove degradation products of cinchonidine. An aliquot of the washed ethylene dichloride is then shaken with a saturated methyl orange solution at pH 5. The excess methyl orange is removed, the ethylene dichloride acidified, and the concentration of the highly colored acid salt of methyl orange measured photometrically.

The procedure is specific to the extent that it is subject to negligible interference from normally occurring substances or the degradation products of cinchonidine which are found in biological material. Recoveries of added cinchonidine average 95 per cent of the theoretical, with total quantities of cinchonidine as low as 5  $\gamma$ . The variation in the recovery of these small amounts is usually less than 5 per cent.

#### *Reagents—*

1. Standard solution of cinchonidine, 100 mg. per liter. 100 mg. of the free base are dissolved in exactly 1 liter of 0.1 N  $H_2SO_4$ . This solution is stable when stored in the refrigerator. More dilute working standards are prepared daily by dilution with water.

2. 1 N NaOH.

### 3. 2.5 N NaOH.

4. Ethylene dichloride. Commercial grades of ethylene dichloride are sometimes contaminated with extraneous material which combines with the methyl orange in the routine procedure. These may have their origin in rubber, cork, or other organic material that comes in contact with the solvent and may be removed by shaking the ethylene dichloride with one-fifth the volume of 1 N HCl. This is followed by a wash with water. It is advised that all ethylene dichloride be treated prior to use in the estimation.

5. Alcoholic KOH solution, 0.1 N KOH in 20 per cent ethyl alcohol.

6. Methyl orange solution. This is prepared as a saturated solution in 0.5 M boric acid. An excess of the sodium salt of methyl orange is added to the boric acid solution, and the mixture is heated gently, cooled to room temperature, and filtered. The solution is washed several times by shaking with an equal volume of ethylene dichloride. Boric acid rather than a salt buffer is used to poise the pH of the solution at about 5.0, since inorganic salts lower the solubility of methyl orange.

7. Alcoholic  $\text{H}_2\text{SO}_4$  solution, 2 ml. of concentrated  $\text{H}_2\text{SO}_4$  in 100 ml. of absolute alcohol.

*Procedure for Plasma*—Add 1 to 5-ml. of plasma and 1 ml. of 1 N NaOH to 20 ml. of ethylene dichloride in a 60 ml. glass-stoppered bottle and shake for 5 minutes, preferably on a shaking apparatus.<sup>1</sup> Decant the contents of the bottle into a 40 ml. round bottomed tube and centrifuge for 10 minutes at 2500 R.P.M. to break the emulsion. Remove the supernatant aqueous layer by aspiration.<sup>2</sup> Return the ethylene dichloride solution to a 60 ml. glass-stoppered bottle (the original thoroughly rinsed out bottle may be used), restraining any coagulum present with a stirring rod. Add an equal volume of the alcoholic KOH solution and shake for 10 minutes.<sup>3</sup> Transfer the contents to a 40 ml. tube (the original rinsed out tube may be used) and centrifuge for 1 minute at 2500 R.P.M. Remove the supernatant aqueous layer completely by aspiration and decant the ethylene dichloride into a 60 ml. glass-stoppered bottle (the original thoroughly rinsed out bottle may be used).<sup>4</sup> Add 0.5 ml. of methyl orange reagent and shake for 5 minutes.

<sup>1</sup> The pH of the aqueous phase during the initial extraction is not critical, provided it is higher than 9.

<sup>2</sup> A solid emulsion sometimes forms in the ethylene dichloride. This may be broken by vigorous stirring with a glass rod. A second centrifugation will then produce a clean separation of the two phases.

<sup>3</sup> This step serves to remove a blank from plasma. It is believed that this blank is caused by the absorption of methyl orange on small particles floating in the solvent and that this particulate matter is removed by the alcoholic KOH wash.

<sup>4</sup> It is important that all traces of alkali be removed, since any that remains will alter the pH of the methyl orange reagent added in the next step of the procedure. This constitutes a major source of error in the routine application of the procedure.

Decant into a 25 ml. test-tube and centrifuge for 5 minutes at 3000 R.P.M. Carefully remove all the supernatant layer by aspiration, decant the ethylene dichloride phase into a 25 ml. test-tube, and recentrifuge for 5 minutes. Pipette 10 ml. of the ethylene dichloride into a colorimeter tube containing 1 ml. of the alcoholic  $H_2SO_4$  and mix thoroughly. Read in the colorimeter with a filter having a maximal transmission at 540  $m\mu$ .

A reagent blank in which water is substituted for plasma is run through the same procedure and is used for setting the instrument to 100 per cent transmission. This reagent blank should not give a transmission of less than 97 when ethylene dichloride plus the alcoholic  $H_2SO_4$  is used to set the instrument to 100 (Evelyn photoelectric colorimeter).

*Procedure for Urine*—Add 1 ml. of diluted urine (*i.e.*, to contain 10 to 40  $\gamma$  of cinchonidine) and 1 ml. of 1 N NaOH to 20 ml. of ethylene dichloride in a 60 ml. glass-stoppered bottle, and shake for 5 minutes. Allow the two phases to separate and remove the aqueous supernatant layer by aspiration. Add an equal volume of 10 per cent NaOH and again shake for 5 minutes. Allow the phases to separate and remove the aqueous layer as above. Then add a few ml. of water and mix with the bottle contents to dilute any residual alkali. Decant into a 40 ml. round bottomed tube and centrifuge for 2 minutes. Remove the supernatant layer completely by aspiration and return the ethylene dichloride solution to the original thoroughly rinsed out bottle.<sup>4</sup> Add 0.5 ml. of methyl orange reagent and proceed as for plasma.

*Procedure for Feces*—Add 20 ml. of concentrated HCl to the sample of feces and dilute to a known volume with water. Shake until a homogeneous mixture is obtained. A suitable aliquot may be analyzed by the procedure described for plasma. The wash with alcoholic KOH is not necessary.

*Standard Curve*—Standards are prepared by taking 1 ml. of standard solution, adding 1 ml. of 1 N NaOH and 20 ml. of ethylene dichloride, and handling in the same manner as for the plasma determination. The wash with alcoholic KOH is omitted. As noted above, a reagent blank is run through the same procedure and is used for setting the instrument to 100 per cent transmission. A permanent standard curve is constructed on semilogarithmic paper, the per cent transmission being plotted against micrograms of alkaloid per ml. of ethylene dichloride. This is a highly reproducible linear relationship; so that standards need not be run together with each set of determinations. A transmission of 52 is obtained on the Evelyn photoelectric colorimeter for a concentration of 1  $\gamma$  of cinchonidine per ml. of ethylene dichloride.

### Results

Table I contains a summary of recoveries of cinchonidine added to plasma and urine. These results indicate that the sensitivity and precision of the

method are quite adequate for use in an appraisal of the therapeutic efficacy of cinchonidine when used as an antimalarial. Equally good results have been obtained in the case of the other common cinchona alkaloids.

Repeated analyses run on individual samples of plasma and urine over a period of several weeks yield highly reproducible results. It may be con-

TABLE I  
*Recovery of Cinchonidine Added to Plasma and Urine*

Plasma			Urine		
Cinchonidine added	Cinchonidine found	Recovery	Cinchonidine added	Cinchonidine found	Recovery
$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	per cent
5	5.4	108	20	20.0	100
	4.8	96		20.8	104
	5.2	104		19.2	96
10	9.6	96	40	19.1	96
	10.0	100		20.2	101
	9.8	98		20.3	102
	9.5	95		20.2	101
	9.5	95		20.4	102
	10.2	100		20.6	103
	9.6	96		20.4	102
	9.6	96		20.0	100
	9.7	97		41.9	105
	10.2	102		40.7	102
20	20.0	100		39.8	100
	19.6	98		39.0	97
	21.2	106		41.8	105
	20.2	101		40.2	101
	20.0	100		40.3	101
	20.0	100		41.4	104
	19.6	98			
	21.6	108			
	21.2	106			
	21.0	105			
30	30.3	101			
	29.4	98			
	31.0	103			

cluded from this finding that the cinchona alkaloids are stable in biological material when stored in the refrigerator.

#### *Appraisal of Specificity*

There is a negligible amount of material in normal plasma or urine which reacts as cinchonidine in the analytical procedures described above. Consequently, specificity depends upon the completeness with which the products of the metabolism of the drug are excluded. This has been ex-



amined by a technique described below which yields information both on the extent of the interference of such products in the general method and how they may be excluded in a revised procedure. Such an approach to an examination of specificity is generally applicable in the development of methods, and is particularly valuable when, as in the present method, a non-selective reaction is used.

The examination of specificity which has been devised is analogous to that utilized by Craig in an examination of certain procedures recommended for the estimation of quinacrine (3). The distribution coefficients of quinacrine and apparent quinacrine extractable from biological material by ethylene dichloride were determined in a two phase system consisting of ethylene dichloride and water-methyl alcohol solutions in varying proportions buffered to a constant pH. Simple ethylene dichloride extracts of plasma and urine were found to contain material with different distribution coefficients than quinacrine. Further study showed that washing of the initial ethylene dichloride extracts with strong alkali removes all fluorescent material other than that which has identical distribution coefficients to those of quinacrine. It was in consequence of these findings that an alkaline wash was introduced in the routine estimation of quinacrine (4).

The present technique differs from that of Craig in that the distribution coefficients of the drug are determined in an ethylene dichloride-water system at various pH values of the aqueous phase. The distribution of a substance in such a system, at constant temperature and at a particular pH, is dependent upon the relative concentrations of the various molecular species in the aqueous phase, as reflected in the dissociation constants, and the relative solubilities of each molecular species in the two phases of the system. Both properties may be rigidly defined, being physical constants. A drug and its metabolic products will have the same distribution coefficients with varying pH of the aqueous phase only if they have the same physical constants. This is an unlikely possibility. However, proof of specificity is always one of probability, and should equivocal evidence be forthcoming from an initial examination, further study with another organic solvent is advisable.

Such a method has an advantage in that it not only indicates the extent to which a procedure is specific but also gives information relative to the solubility characteristics of the interfering metabolic products of the drug. The latter type of information may be readily applied in the revision of the original procedure so as to exclude the interfering substances. This is usually possible by an adjustment of the pH of the aqueous phase in the initial extraction or by the inclusion of a suitable wash of the organic solvent applied subsequently in the procedure.

To serve as an illustration, the principle of the procedure is described in

relation to the examination of the specificity of the analytical procedure as proposed for the estimation of cinchonidine.

#### EXPERIMENTAL

15 ml. of diluted urine containing 100 to 200  $\gamma$  of apparent cinchonidine and 15 ml. of 1 N NaOH were added to a 500 ml. glass-stoppered Pyrex bottle containing 300 ml. of ethylene dichloride and shaken for 10 minutes. The mixture was transferred to large centrifuge tubes, centrifuged, and the aqueous phase completely removed by aspiration.<sup>4</sup> Two 15 ml. aliquots of the ethylene dichloride phase were set aside for direct analysis ( $C_1 + C_2$ ). 15 ml. aliquots of buffer solutions (pH 5 to 14 in units of 1) were added to 15 ml. aliquots of the remaining ethylene dichloride in 60 ml. glass-stoppered Pyrex bottles.<sup>5</sup> The mixtures were shaken for 1 hour and then centrifuged. The aqueous phase of each sample was completely removed by aspiration and the ethylene dichloride decanted into 60 ml. glass-stoppered Pyrex bottles containing 0.5 ml. of the methyl orange reagent.<sup>4</sup> The concentration of apparent cinchonidine in each sample was then determined as in the analytical procedure described above ( $C_1$ ).

Results of a comparison of a solution of pure cinchonidine with the apparent cinchonidine extracted from urine as described above are shown in Table II. Distribution is described as  $C_1/(C_1 + C_2)$ , as a matter of convenience, where  $C_1$  is the concentration in the organic phase,  $C_2$  that in the aqueous phase. It is apparent from a consideration of the data that the ethylene dichloride extract of urine contains methyl orange-reacting material with solubility characteristics that differ from those of pure cinchonidine. The urine extract contains material which is water-soluble at the higher pH values. The differences observed are not due to the manipulations in the procedure, since cinchonidine added to urine or other biological material is extractable unchanged with ethylene dichloride. These data carry the suggestion that the introduction of an alkali wash of the ethylene dichloride extract will confer additional specificity on the analytical procedure.

The effect of the introduction of such a wash was studied as follows: A sample of urine was treated as described above until the urine and ethylene dichloride had been shaken for 10 minutes in the initial extraction. The two phases were allowed to separate and the aqueous layer removed. An equal volume of 10 per cent NaOH was added and the mixture shaken for

<sup>5</sup> The distribution ratios at the different pH values are reproducible at constant temperature. However, it is not necessary to maintain a constant given temperature, provided the determinations for any examination contain a control series of samples of the pure drug. Such a procedure also corrects for minor variations in the pH of the buffers used.

10 minutes. The phases were again allowed to separate and the aqueous layer removed by aspiration. A few ml. of water were added and mixed with the bottle contents to dilute any residual alkali. The mixture was then transferred to large centrifuge tubes, centrifuged, and the aqueous phase completely removed by aspiration.<sup>4</sup> Two 15 ml. aliquots of the ethylene phase were set aside for direct analysis ( $C_1 + C_2$ ). The remainder

TABLE II

*Distribution of Cinchonidine and Apparent Cinchonidine between Water and Ethylene Dichloride at Various pH Values*

The distribution ratios given as a function of the various pH values are expressed as the ratio of the concentration of drug remaining in the organic phase to total drug after shaking with the appropriate buffer. This ratio in terms of symbols noted in the text is expressed as  $C_1/(C_1 + C_2)$ , where  $C_1$  is the concentration in ethylene dichloride, and  $C_2$  is the concentration in water.

pH	Aqueous control (a)	Plasma			Urine	
		Control (b)	Patient 1 (c)	Patient 2 (d)	Patient 1, before alkali wash (e)	Patient 1, after alkali wash (f)
5	0.02	0.04	0.04	0.04	0.02	0.03
6	0.15	0.15	0.18	0.17	0.08	0.18
7	0.64	0.63	0.65	0.62	0.29	0.65
8	0.92	0.96	0.96	0.91	0.57	0.91
9	0.97	0.99	1.00	0.98	0.64	1.00
10	0.98	0.98	1.00	0.99	0.72	1.02
11	0.97	0.99	1.02	0.98	0.72	1.03
12	0.99	0.97	0.99	1.00	0.66	1.00
13	1.00	0.98	1.01	1.01	0.56	1.03
14	0.99	0.99	1.02	0.99	0.54	1.03

Column (a), cinchonidine added to water and extracted into ethylene dichloride; (b), cinchonidine added to plasma and extracted into ethylene dichloride; (c) and (d), apparent cinchonidine from plasma of patients, extracted into ethylene dichloride; (e), apparent cinchonidine from urine extracted into ethylene dichloride, before alkali wash; (f), apparent cinchonidine from urine extracted into ethylene dichloride, after alkali wash.

was shaken with a series of buffer solutions as described above. The results of the examination are summarized in Table II. The data indicate that, as expected, the treatment with an alkali wash removes certain degradation products of cinchonidine from the ethylene dichloride phase, leaving behind a substance with solubility characteristics identical to those of cinchonidine.<sup>6</sup>

<sup>6</sup> The degradation products of cinchonidine and cinchonine are quite soluble in alkali, one alkaline wash of the ethylene dichloride extract sufficing for their removal from both urine and plasma. The degradation products of quinine and quinidine are

A similar study was made of the ethylene dichloride extracts of plasma.<sup>7</sup> This indicates that the wash with the alcoholic KOH solution, which is included in the routine procedure, is sufficient to remove completely any degradation products which are present.

#### DISCUSSION

Information is at hand to indicate that the general procedure described above is applicable, with modifications, to a large number of alkaline drugs among which are codeine, plasmochin, benzedrine, ephedrine, and demerol. The modifications which will be required for the estimation of any one of these have not been worked out. However, it may be stated that these will vary according to the physical properties of the compound and its concentration in the biological sample. A reaction similar in principle has been used in this laboratory for the estimation of certain organic acids. Here, the acid forms a complex with a basic dye (rosaniline) that is soluble in ethylene dichloride.

A reaction somewhat similar to the methyl orange reaction has been described previously (5). The formation of a chloroform-soluble complex of eosin and quinine was utilized to estimate the concentration of quinine in urine. Eosin has been studied in this laboratory but its use is not advised, since it has a low color index and a relatively high solubility in chloroform and ethylene dichloride. These characteristics preclude its use in the analysis of alkaline drugs at the concentrations commonly observed in plasma. Two other somewhat similar procedures have been described more recently, one to estimate quaternary ammonium compounds (6), the other demerol (7), by forming organic soluble complexes with bromphenol blue. This reaction is not generally useful in its present stage of development for application to plasma owing to a lack of sensitivity.

#### SUMMARY

A simple general method is described which, with minor modifications, can be applied to the development of methods for a large number of alkaloids and synthetic alkaline organic compounds. The method has been applied to the development of an analytical procedure for the estima-

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less soluble in water at a high pH. Consequently several alkaline washings are required for their removal in the analysis of urine in which they occur in high concentration.

<sup>7</sup> Some alcohol is present in the ethylene dichloride extract of plasma as a result of the wash with alcoholic KOH. It is advisable to measure the distribution constants in the absence of alcohol. The cinchonidine is therefore quantitatively extracted from the ethylene dichloride phase into aqueous acid, the latter alkalized, and the cinchonidine finally returned to ethylene dichloride. The alcohol remains behind in the aqueous phase.

tion of the cinchona alkaloids, particularly cinchonidine, for illustrative purposes.

Cinchonidine is isolated from the biological material by an extraction of the free base with ethylene dichloride at an alkaline pH. The ethylene dichloride is washed free of degradation products with alkali and shaken with methyl orange solution. Cinchonidine forms a salt with methyl orange which is soluble in ethylene dichloride and its concentration is estimated by determining the optical density of the ethylene dichloride solution.

A technique is also described which can be used for appraising the specificity of many analytical procedures. The distribution coefficients of the pure and apparent compound are measured in an ethylene dichloride-water system at various pH values of the latter phase. The procedure yields information on the extent to which degradation products of the drug interfere in the estimation and the solubility characteristics of the interfering substances. The latter type of information is used in devising modifications of the basic procedure which introduce the desired specificity.

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## LETTERS TO THE EDITORS

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### THE PREVENTION BY CAPRYLATE OF UREA AND GUANIDINE DENATURATION OF SERUM ALBUMIN

Sirs:

Low concentrations of sodium caprylate and related compounds markedly increase the thermal stability of human and bovine serum albumin,<sup>1</sup> and prevent the rapid rate of enzyme digestion of serum albumin otherwise induced by urea denaturation.<sup>2</sup> Sulfate, as well as other inorganic anions, decreases SH group liberation by urea or guanidine.<sup>3</sup> The denaturation of serum albumin by urea or guanidine hydrochloride results in a pronounced viscosity increase, attributable to a more unfolded or extended albumin molecule.<sup>4</sup> In this note viscosity studies are reported which have shown that low concentrations of caprylate or relatively high concentrations of sulfate prevent the marked extension of the albumin molecule which otherwise occurs with solutions of albumin in 6 M urea or 2.5 M guanidine hydrochloride, and, furthermore, effect a considerable decrease in the viscosity of serum albumin previously denatured in urea or guanidine solutions.

Typical results of viscosity measurements are given in the table. The values represent (1) denatured albumin, albumin in denaturing solution for 15 minutes; (2) stabilized albumin, caprylate or sulfate added before the albumin; and (3) partially regenerated albumin, caprylate or sulfate added 15 minutes after the albumin addition and the viscosity determined after an additional 15 minutes. The viscosity decrease following caprylate addition to albumin denatured in urea is much more pronounced than the decrease noted in enzyme digestion rates.<sup>2</sup> A caprylate concentration of 0.005 M was sufficient for maximum protection in 6 M urea. Data from ultrafiltration experiments showed that with 0.0050 M caprylate, about 0.0024 M caprylate was bound by the albumin, or about 9 molecules of caprylate per molecule of albumin. Weight intrinsic viscosities, cal-

<sup>1</sup> Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **153**, 589 (1944). Boyer, P. D., and Lum, F. G., unpublished data.

<sup>2</sup> Rice, R. G., Ballou, G. A., Boyer, P. D., Luck, J. M., and Lum, F. G., *J. Biol. Chem.*, **158**, 609 (1945).

<sup>3</sup> Burk, N. F., *J. Phys. Chem.*, **47**, 104 (1943). Greenstein, J. P., *J. Biol. Chem.*, **130**, 519 (1939).

<sup>4</sup> Neurath, H., Cooper, G. R., and Erickson, J. O., *J. Biol. Chem.*, **142**, 249 (1942).

culated from viscosities of 0 to 2 gm. per cent albumin solutions, were 4.4, 5.2, and 11.2 for albumin in solutions with no urea, 6 M urea and 0.02 M caprylate, and 6 M urea with no caprylate, respectively. The slight difference in relative viscosities of caprylate-stabilized albumin with and without urea present may be due to either a slight denaturation of the albumin or to a solvation of the albumin by about 0.18 to 0.27 gm. of urea per gm. of protein.

With the albumin in 2.5 M guanidine hydrochloride, the concentration of caprylate or of sulfate necessary for maximum effect was 0.025 and 0.050 M respectively. Hence, caprylate was about 20 times more effective

*Effect of Sodium Caprylate and Sodium Sulfate on Viscosity of Human Serum Albumin in Urea and Guanidine Solutions*

The solutions contained 2.0 gm. per cent albumin, 0.01 M phosphate buffer, 0.025 M sodium chloride, and were at pH 6.9 to 7.1. The viscosities, determined with an Ostwald type viscometer at  $30^{\circ} \pm 0.01^{\circ}$ , are expressed as the viscosity relative to the solution without albumin. The relative viscosity of 2 gm. per cent albumin in phosphate buffer was 1.090.

Treatment of sample	Albumin in 6 M urea		Albumin in 2.5 M guanidine hydrochloride			
	Molarity of Na caprylate	Relative viscosity	Molarity of Na caprylate	Relative viscosity	Molarity of Na <sub>2</sub> SO <sub>4</sub>	Relative viscosity
Denatured albumin	0	1.278	0	1.281	0	1.281
Stabilized "	0.025	1.104	0.025	1.096	0.50	1.105
Partially regenerated albumin...	0.025	1.131	0.025	1.115	0.50	1.132

than sulfate. The effect of guanidine hydrochloride on human serum albumin was much greater than that of urea, and at a concentration of 6 M was not prevented by any concentration of caprylate.

The prevention of heat or urea denaturation by small amounts of caprylate indicates that caprylate exerts its effect by combination with certain groups or areas on the albumin molecule, and that the same bonds are involved in the initial phases of denaturation by heat or urea.

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Department of Chemistry  
Stanford University  
California

PAUL D. BOYER

## THE LYSINE CONTENT OF CASEIN AND ZEIN

Sirs:

In connection with an investigation on the metabolism of lysine,<sup>1</sup> an analysis of casein by the decarboxylase method of Gale and Epps<sup>2</sup> was reported. The figure of 6.7 per cent found is lower than the values reported by Zittle and Eldred<sup>3</sup> who also used the decarboxylase technique and that by Dunn *et al.*<sup>4</sup> employing a microbiological assay method. It was erroneously stated in our paper that the figure was corrected for ash and volatile matter. The casein sample used contained 2.6 per cent ash and 7.8 per cent volatile matter and the corrected lysine content of casein on an ash- and water-free basis is therefore 7.5 per cent, a value which is in good agreement with that found by the workers mentioned above. We have now repeated lysine estimations with the decarboxylase method on several samples of casein using hydrolysis with HCl and obtained values of between 7.5 and 7.8 per cent of lysine on a water- and ash-free basis. The value given by Dunn *et al.* is slightly higher and it may therefore be assumed that casein contains between 7.5 and 8 per cent of lysine.

The amount of lysine present in zein is of special interest in connection with the use of this protein in dietary experiments in which the absence of lysine is desired. Zein was hydrolyzed with 10 N H<sub>2</sub>SO<sub>4</sub> for 18 hours and the hydrolysate neutralized with Ba(OH)<sub>2</sub>. The BaSO<sub>4</sub> precipitate was repeatedly extracted with boiling water; the combined solutions were concentrated and used for the estimation of lysine. The amounts found were negligible, even after the hydrolysates has been freed of most of the glutamic acid and some of the more insoluble monoamino acids. This is in agreement with the findings of Zittle and Eldred.<sup>3</sup> The minute and sluggish evolution of CO<sub>2</sub> found with some zein samples may be due either to contamination with other proteins or to the presence of hydroxylysine.

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<sup>1</sup> Neuberger, A., and Sanger, F., *Biochem. J.*, **38**, 119 (1944).

<sup>2</sup> Gale, E. F., and Epps, H. M. R., *Nature*, **152**, 327 (1943); *Biochem. J.*, **38**, 232 (1944).

<sup>3</sup> Zittle, C. A., and Eldred, N. R., *J. Biol. Chem.*, **156**, 401 (1944).

<sup>4</sup> Dunn, M. F., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 715 (1944).





## ISOLATION OF A URINARY STEROID WITH AN OXYGEN ATOM AT CARBON 11

Sirs:

A compound with an oxygen atom at C 11 of the steroid nucleus has been isolated from the urine of seven patients with disease of the adrenal cortex. There were three women with tumors, three female pseudohermaphrodites with hyperplasia, and one young boy with hyperplasia. This compound was obtained from the alcoholic ketonic fraction of the neutral extract of the acidified, boiled urine and was eluted from a chromatographic column of alumina with carbon tetrachloride containing 0.5 per cent by volume of absolute alcohol. It has the formula  $C_{19}H_{30}O_3$  (calculated, C 74.44, H 9.87; found, C 74.24, H 10.19), melts at 197–198°, and  $[\alpha]_D^{25} = +96.6^\circ \pm 2^\circ$ . When it was heated at 90° for 30 minutes with acetic anhydride and pyridine, it formed a monoacetate,  $C_{21}H_{32}O_4$  (calculated, C 72.38, H 9.26; found, C 72.77, H 9.17), which melted at 240–242°. The original substance did not give a precipitate with digitonin in 90 per cent methanol. It formed a yellow dinitrophenylhydrazone which melted at 250–251°.

Oxidation with chromic acid gave a product which was found to be identical with androstane-3,11,17-trione (calculated for  $C_{19}H_{26}O_3$ , C 75.46, H 8.67; found, C 75.98, H 9.00). After recrystallization from dry ether it melted at 178–179°;  $[\alpha]_D^{28} = +151^\circ \pm 3^\circ$ . Androstane-3,11,17-trione prepared from an adrenal steroid by Mason, Hoehn, and Kendall<sup>1</sup> melted at 178–179°;  $[\alpha]_D^{28} = +152^\circ \pm 2^\circ$ . A mixture of androstane-3,11,17-trione with the oxidation product of the urinary compound also melted at 178–179°. The oximes of the oxidation product and of androstane-3,11,17-trione both melted at 264–268° (corrected) with decomposition and a mixture of the two also melted at 264–268° (corrected).

Spectrophotometric analysis of the colors produced by the urinary substance and its oxidation product in the Zimmermann reaction gave curves with maximal absorption at 520 m $\mu$ , similar in other respects to the curves obtained with known 17-ketosteroids. The urinary compound produced 73 per cent and the oxidation product 115 per cent as much color as dehydroisandrosterone on a molecular basis.

These results speak for a formulation of the urinary compound as 11 $\beta$ -hydroxyandrosterone (androstane-3 $\alpha$ ,11 $\beta$ -diol-17-one). The possibility of a ketone group at position 11 cannot be excluded with the evidence available. However, the analyses favor the conclusion that there are two hydroxyl groups present, one of which is not readily acetylated. Von Euw and Reichstein<sup>2</sup> have isolated from adrenal extracts androstane-

<sup>1</sup> Mason, H. L., Hoehn, W. M., and Kendall, E. C., *J. Biol. Chem.*, **124**, 459 (1938).

<sup>2</sup> von Euw, J., and Reichstein, T., *Helv. chim. acta*, **24**, 879 (1941).

3 $\beta$ ,11 $\beta$ -diol-17-one, which melts at 236° and which forms only the 3-monoacetate with acetic anhydride in pyridine. Reichstein<sup>3</sup> has also oxidized this substance as well as other adrenal steroids to androstane-3,11,17-trione.

The urinary compound conceivably could be a metabolic product of any of the adrenal steroids which have an oxygen atom at C 11 but it is more likely the product of the metabolism of compounds which also have a hydroxyl group at C 17 such as 17-hydroxycorticosterone.

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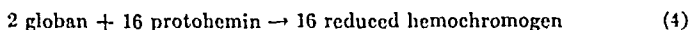
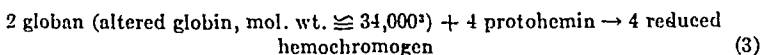
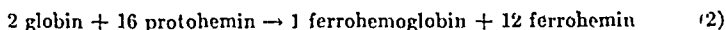
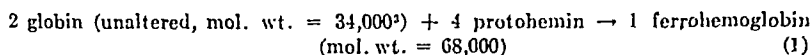
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<sup>3</sup> Reichstein, T., *Helv. chim. acta*, **19**, 402 (1936).

# UNALTERED GLOBIN, AND CRYSTALLINE, SYNTHETIC (RECONSTITUTED) MYOGLOBIN

Sirs:

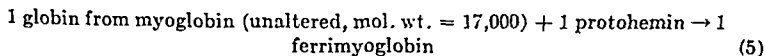
The degree of alteration of isolated globin may be determined by the spectrophotometric analysis of synthetic products formed from globin and protohemin. At weakly alkaline pH, with  $\text{Na}_2\text{S}_2\text{O}_4$ , the following reactions occur.<sup>1,2</sup>



These equations state that 1 mole of native globin (mol. wt. = 34,000) combines in the presence of excess hemin with only 2 hemin units (Reaction 2), whereas altered globin (same molecular weight) may combine with as many as 8 units of hemin<sup>2</sup> (Reactions 3 and 4).

Even the best reconstituted hemoglobins, obtained by Reaction 1, are less stable<sup>3,4</sup> than natural hemoglobin, and differ from the latter in electrophoretic mobility<sup>3</sup> and solubility after electrodialysis.<sup>3</sup> In the writer's hands fresh acid globin preparations, made by the splitting of hemoglobin in HCl-acetone,<sup>5</sup> yield with hemin and  $\text{Na}_2\text{S}_2\text{O}_4$  mixtures of 70 to 60 per cent of ferrohemoglobin and 30 to 40 per cent of reduced hemochromogen (a combination of Reactions 1 and 3).

Since myoglobin is more stable to alkali than hemoglobin,<sup>6</sup> the preparation of the globin from the muscle pigment was undertaken with the hope of securing a relatively stable and unaltered globin. This has been realized, and has permitted a successful synthesis of ferrimyoglobin, presumably identical with the corresponding form of the natural pigment, according to the reaction,



<sup>1</sup> Drabkin, D. L., *Proc. Soc. Exp. Biol. and Med.*, **41**, 225 (1939).

<sup>2</sup> Drabkin, D. L., *Federation Proc.*, **2**, 61 (1943).

<sup>3</sup> Gralén, N., *Biochem. J.*, **33**, 1907 (1939).

<sup>4</sup> Roche, J., and Combette, R., *Bull. Soc. chim. biol.*, **19**, 627 (1937).

<sup>5</sup> Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **13**, 469 (1929-30). Schenck, E. G., *Arch. exp. Path. u. Pharmacol.*, **150**, 160 (1930).

<sup>6</sup> Theorell, H., *Biochem. Z.*, **262**, 1 (1932); **268**, 46 (1934).

Steps in the preparation and synthesis follow: (a) Crystalline (orthorhombic<sup>7</sup>) ferrimyoglobin was isolated<sup>6,8</sup> from horse heart. (b) A salt-free pigment solution was secured by dialysis at 0°. (c) By means of the lyophile process the myoglobin was recovered in dry state, and from the latter a concentrated solution (~4.0 mM per liter) was obtained. (d) The globin was split off in chilled HCl-acetone (10 volumes of solvent, 1 volume of pigment solution, pH 1.9), washed once with acid acetone, and two times with unacidified acetone. These procedures and subsequent synthesis were performed quantitatively, at room temperature, in centrifuge tubes. (e) The acid globin was dissolved in water, and to this solution was added in 0.03 M NaOH an equimolecular quantity of crystalline  $\alpha$ -chlorohemin, prepared from dog hemoglobin.<sup>9</sup> The still acid mixture was brought to pH 7.2, sufficient alkali being used to dissolve the isoelectric precipitate, at pH 6 to 7, of synthetic ferrimyoglobin.

Spectrophotometry upon aliquots of the stable, neutralized solution (concentration of pigment  $\cong$  1 mM per liter) revealed complete recovery of the protein as ferrimyoglobin, identical with the natural substance in respect to absorption constants and characteristic wave-length locations of maxima, in the presence of  $\text{CN}^-$ ,  $\text{Na}_2\text{S}_2\text{O}_4$ ,  $\text{Na}_2\text{S}_2\text{O}_4 + \text{CO}$ , and  $\text{Na}_2\text{S}_2\text{O}_4 + \text{O}_2$ .

The synthetic ferrimyoglobin has been successfully crystallized in the habit of sheaves of capillary prisms (needles), identical with the characteristic crystal habit of the natural pigment.

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<sup>7</sup> Drabkin, D. L., *Am. J. Med. Sc.*, **209**, 268 (1945).

<sup>8</sup> Morgan, V. E., *J. Biol. Chem.*, **112**, 557 (1935-36).

<sup>9</sup> Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 89 (1935-36).

## ENZYMATIC SYNTHESIS OF A NUCLEOSIDE

Sirs:

The purine nucleosidases have been thought to split nucleosides into purines and free ribose (or desoxyribose). Evidence is herein presented that 1-phosphoribose rather than free ribose is liberated.

It has been reported that nucleosidases are activated by phosphate and arsenate.<sup>1</sup> We have studied the action of a specific inosine nucleosidase prepared from rat liver and have found that the enzyme is a phosphorylase which catalyzes the reversible reaction, inosine (hypoxanthine riboside) + phosphate  $\rightleftharpoons$  hypoxanthine + ribose-1-phosphate. The equilibrium greatly favors synthesis of the nucleoside.

The liberation of hypoxanthine from inosine in the presence of nucleosidase requires phosphate. 1 mole of phosphate is taken up for each mole of hypoxanthine liberated. The reaction does not proceed very far, however, except in the presence of large amounts of phosphate, or unless the hypoxanthine formed is oxidized to uric acid by addition of xanthine oxidase. In the latter case the reaction goes to completion and 1 mole of uric acid is formed for each mole of P which disappears. Incubation of hypoxanthine plus ribose with xanthine oxidase and nucleosidase does not cause a change in the inorganic phosphate.

It has been possible to isolate as a barium salt a new phosphoric ester which is formed by the action of the nucleosidase on inosine. The ester is extraordinarily labile to strong acids. Incubation for 1 minute at 30° in 0.3 N HCl is sufficient to hydrolyze 50 per cent of it, and after 15 minutes incubation in acid, the ester is completely hydrolyzed. The ester contains approximately equimolar amounts of pentose and of labile phosphate (0.9 micromole of pentose per micromole of labile P). No free aldose groups are present. However, after hydrolysis approximately one aldose group is found per mole of labile P (0.9 micromole of aldopentose for 1 micromole of labile P). The ester must, therefore, be characterized as a pentose-1-phosphate, and since it is formed from a ribose nucleoside it seems justified to assume that the ester is ribose-1-phosphate.

If ribose-1-phosphate is incubated with hypoxanthine and nucleosidase, inosine is synthesized and an equimolar amount of inorganic phosphate is formed. Thus, 0.58 micromole of hypoxanthine plus 0.58 micromole of ribose-1-phosphate incubated for 20 minutes with 150  $\gamma$  of nucleosidase gave the following result: inorganic P liberation 0.36 micromole; hypoxanthine disappearance, 0.39 micromole; inosine synthesis, 0.39 micromole. The controls in which one of the components was added after denaturation of the

<sup>1</sup> Klein, W., *Z. physiol. Chem.*, **231**, 125 (1935).

enzyme contained the amounts of hypoxanthine and ribose-1-phosphate originally added.

The equilibrium (reached within 20 minutes) favors the formation of inosine; at least 70 per cent of the free purine and ribose-1-phosphate is converted to inosine and inorganic phosphate at pH 6.5. The synthesis of inosine would probably proceed farther if all traces of inorganic phosphate were removed, since with equimolar amounts of inosine and phosphate as starting material it was found that only 10 per cent of the riboside undergoes phosphorolysis at pH 6.5.

The technique involved in these experiments was as follows: Inosine and hypoxanthine were determined optically at 290 m $\mu$  as described in a previous paper.<sup>2</sup> Phosphate was determined by a micromethod devised by Dr. O. H. Lowry<sup>3</sup> in which it is possible to determine inorganic phosphate in the presence of a highly acid labile phosphate ester. Pentose was determined colorimetrically<sup>4</sup> and aldose titrimetrically.<sup>5</sup>

We are suggesting that the enzyme catalyzing the equilibrium reaction be called nucleoside phosphorylase, in analogy with Cori's polysaccharide phosphorylase and Doudoroff's disaccharide phosphorylase.

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<sup>2</sup> Kalckar, H. M., *J. Biol. Chem.*, **158**, 313 (1945).

<sup>3</sup> Lowry, O. H., unpublished experiments.

<sup>4</sup> Mejbaum, W., *Z. physiol. Chem.*, **258**, 117 (1939).

<sup>5</sup> Macleod, M., and Robinson, R., *Biochem. J.*, **23**, 517 (1929).

## THE ISOLATION OF $\alpha$ -ESTRADIOL FROM THE URINE OF STALLIONS\*

Sirs:

Since the discovery<sup>1</sup> that the urine of stallions contains large quantities of estrogenic material, a number of reports of the isolation of estrone from this source have appeared. A search of the literature does not, however, reveal any attempt to investigate the non-ketonic estrogenic fraction of such urine, although Beall<sup>2</sup> isolated  $\alpha$ -estradiol from stallion testes in a yield of 0.21 mg. per kilo of fresh tissue.

The present writer has investigated four specimens of stallion urine and has found that a major proportion of the estrogenic activity appears in the weakly phenolic, non-ketonic fraction of the urine extract and is apparently due to estradiol. In one fresh sample of pooled urine from several stallions, assaying about 150,000 rat units of estrogenic activity per liter, the weakly phenolic, non-ketonic fraction accounted for more than 90 per cent of the activity. A similar figure was obtained from the concentrate of another pooled sample of stallion urine. The estradiol content of two urine specimens, collected several months apart from a single stallion, accounted for 42 and 44 per cent, respectively, of the total activity. One of these specimens assayed 57,000 rat units per liter; the other assayed only 4400 rat units per liter.

Estradiol was isolated as the di- $\alpha$ -naphthoate in good yield from the two more active preparations. The fresh, pooled urine specimen yielded chemically pure, crystalline estradiol-di- $\alpha$ -naphthoate in an amount equivalent to 5 mg. of free estradiol per liter of urine. The crystalline products were proved to be estradiol-di- $\alpha$ -naphthoate by melting point, by melting point when mixed with authentic  $\alpha$ -estradiol-di- $\alpha$ -naphthoate, by elementary analysis, and by comparison of the weight of the rat unit to that of the mouse unit of the free estradiol.

To the extent of the writer's knowledge, these findings show certain

\* Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation.

<sup>1</sup> Zondek, B., *Nature*, **133**, 209 (1934). Haussler, E. P., *Helv. chim. acta*, **17**, 531 (1934).

<sup>2</sup> Beall, D., *Biochem. J.*, **34**, 1293 (1940).



values determined experimentally by direct integration of the patterns agreed well with the known composition of the mixture, as can be seen from results given in Table I, and indicate that correct analyses in buffers of low ionic strength can be made by simple integration of the diagrams.

The study was extended to analyses of swine serum. In barbital buffer of pH 8.6 and 7.8, insignificant variations in percentage composition were observed with change in ionic strength, as shown in Table II.

TABLE II  
*Swine Serum\**

Protein concentration	pH	Ionic strength	Albumin concentration	
			Ascending side	Descending side
<i>per cent</i>		$\mu$	<i>per cent</i>	<i>per cent</i>
3.91	7.8	0.1	38.7	39.8
2.58	8.6	0.1	36.4	36.7
3.89	8.6	0.25	37.3	36.9
3.19	8.6	0.40	35.8	35.8

\* All of these analyses were made on the same pool of swine sera.

The results of these experiments show that, with the electrolytes here employed, estimation of the percentage component composition of an artificial mixture of proteins can be made with high accuracy by means of electrophoretic analysis. In the experiments with swine serum there was little evidence of dependence of percentage composition on ionic strength within the limits investigated. From these results it seems clear that Svensson's conclusions in these respects are not wholly applicable to the general problem of electrophoretic analysis.

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# INDEX TO AUTHORS

## A

- Albanese, Anthony A. The utilization of  $\alpha$ -amino acids by man. II. Cystine, 101  
 Anderson, Pearl R. See *MacFadyen*, 107  
 Archibald, Reginald M. Methods for the determination of alloxan, together with observations of certain properties of alloxan, 347

## B

- Baer, John E. See *Brodie and Udenfriend*, 705  
 Ballou, Gerald A. See *Rice, Ballou, Boyer, Luck, and Lum*, 609  
 Banerjee, Sachchidananda. On the hypoglycemic action of alloxan, 547  
 Bartlett, Sister Mary Domitilla. See *Kreke, Bartlett, and Small*, 469  
 Bean, H. W. See *Mitchell, Hamilton, Steggerda, and Bean*, 625  
 Beloff, Ruth L., and Stern, Kurt G. On extracts of carp spleen and their effect on carboxylase and cocarboxylase, 19  
 Benoit, George J., Jr., and Norris, Earl R. Studies on trimethylamine oxide. II. The origin of trimethylamine oxide in young salmon, 439  
 —. See *Norris and Benoit*, 433, 443  
 Bergmann, Max. See *Zamecnik, Lavin, and Bergmann*, 537  
 Bernheim, Frederick, and Bernheim, Mary L. C. The inactivation of tyramine by heart muscle *in vitro*, 425  
 —. See *Darby, DeMeio, Bernheim, and Bernheim*, 67  
 Bernheim, Mary L. C. See *Bernheim and Bernheim*, 425  
 —. See *Darby, DeMeio, Bernheim, and Bernheim*, 67  
 Bina, Albert F. See *Brown, Bina, and Thomas*, 455

- Bischoff, Fritz. Note concerning the reversible inactivation of prolactin, 577  
 —. The temperature coefficient for the denaturation of chorionic gonadotropin by urea, 29  
 Bonsnes, Roy W., and Taussky, Hertha H. On the colorimetric determination of creatinine by the Jaffe reaction, 531  
 Boyer, Paul D. The prevention by caprylate of urea and guanidine denaturation of serum albumin, 715  
 —. See *Rice, Ballou, Boyer, Luck, and Lum*, 609  
 Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B. Studies on vitamins B<sub>12</sub> and B<sub>11</sub> and related substances in chick nutrition, 303  
 Brodie, Bernard B., and Udenfriend, Sidney. The estimation of basic organic compounds and a technique for the appraisal of specificity. Application to the cinchona alkaloids, 705  
 Brown, Elmer B., Bina, Albert F., and Thomas, James M. The use of diazotized *p*-aminoacetophenone in the determination of vitamin B<sub>6</sub> (pyridoxine), 455  
 Brown, Herman. The determination of uric acid in human blood, 601  
 Brown, Jean D. See *Eichelberger and Brown*, 283  
 Bruce, W. F. See *Scott, Norris, Heuser, and Bruce*, 291  
 Buntin, Helen. See *Rathbun and Pace*, 667

## C

- Caldwell, M. J., and Parrish, D. B. The effect of light on the stability of the Carr-Price color in the determination of vitamin A, 181  
 Chalkoff, I. L. See *Friedlander, Chaikoff, and Entenman*, 231  
 Chenkin, Theodore. See *Brodie and Udenfriend*, 705

- Christensen, Halvor N., and Hegsted, D. Mark. The course of the acid hydrolysis of gramicidin, 593
- , Uzman, Lahut, and Hegsted, D. Mark. A note on the amino acid composition of tyrocidine, 279
- Cohen, Seymour S. The enzymatic degradation of thymus nucleohistone, 255
- Cooper, Gerald R. Electrophoretic analysis of mixtures of protein, 727
- Cope, Oliver. See Zamecnik, Stephenson, and Cope, 135
- Cori, Carl F., and Cori, Gerty T. The activity and crystallization of phosphorylase b, 341
- , See *Cori and Cori*, 321
- Cori, Gerty T. The effect of stimulation and recovery on the phosphorylase a content of muscle, 333
- and Cori, Carl F. The enzymatic conversion of phosphorylase a to b, 321
- , See *Cori and Cori*, 341
- Cunningham, Elizabeth, and Snell, Esmond E. The vitamin B<sub>6</sub> group. VI. The comparative stability of pyridoxine, pyridoxamine, and pyridoxal, 491

## D

- Darby, William J., DeMeio, R. H., Bernheim, Mary L. C., and Bernheim, Frederick. The metabolism of phenolic compounds by normal and scorbutic guinea pig liver slices *in vitro*, 67
- Davenport, Horace W. The inhibition of carbonic anhydrase by thiophene-2-sulfonamide and sulfanilamide, 567
- DeMeio, R. H. See *Darby, DeMeio, Bernheim, and Bernheim*, 67
- De Ritter, Elmer. See *Rubin and De Ritter*, 639
- Dill, Wesley. See *Brodie and Udenfriend*, 705
- Drabkin, David L. Unaltered globin, and crystalline, synthetic (reconstituted) myoglobin, 721
- Dutcher, R. Adams. See *Pence, Miller, Dutcher, and Thorp*, 647
- Dziewiatkowski, Dominic D., and Lewis,

Howard B. The metabolism of trimethylacetic (pivalic) and tertiary butylacetic acid. New examples of conjugation with glucuronic acid, 77

## E

- Elchelberger, Lillian, and Brown, Jean D. The fat, water, chloride, total nitrogen, and collagen nitrogen content in the tendons of the dog, 283
- Elvehjem, C. A. See *Briggs, Luckey, Elvehjem, and Hart*, 303
- , See *Krehl and Elvehjem*, 173
- , See *Ruegamer, Michaud, and Elvehjem*, 573
- Entenman, C. See *Friedlander, Chaikoff, and Entenman*, 231

## F

- Floyd, Norman F. See *Weinhouse, Medes, and Floyd*, 411
- Frankston, Jane E. See *Albanese*, 101
- Friedlander, H. D., Chaikoff, I. L., and Entenman, C. The effect of ingested choline on the turnover of plasma phospholipids, 231

## G

- Green, Arda Alden. The diffusion constant and electrophoretic mobility of phosphorylases a and b, 315

## H

- Hamilton, Paul B. Gasometric determination of glutamine amino acid carboxyl nitrogen in plasma and tissue filtrates by the ninhydrin-carbon dioxide method, 375
- , Glutamine: a major constituent of free  $\alpha$ -amino acids in animal tissues and blood plasma, 397
- Hamilton, T. S. See *Johnson, Hamilton, and Mitchell*, 619
- , See *Mitchell, Hamilton, Steggerda, and Bean*, 625
- Hart, E. B. See *Briggs, Luckey, Elvehjem, and Hart*, 303
- Hegsted, D. Mark. See *Christensen and Hegsted*, 593
- , See *Christensen, Uzman, and Hegsted*, 279

- Herrmann, Heinz, and Moses, Sylvia  
G. Content and state of glutathione  
in the tissues of the eye, 33  
— and —. The cytochrome oxidase  
activity of the lens of the bovine eyes,  
47

- Heuser, G. F. See *Scott, Norris,  
Heuser, and Bruce*, 291  
Hill, Edward L. See *Lifson, Lorber,  
and Hill*, 219  
Hinshaw, Howard E. See *Rathbun and  
Pace*, 667

- Hochberg, Melvin, Melnick, Daniel,  
and Oser, Bernard L. Chemical deter-  
mination and urinary excretion of the  
metabolite N<sup>1</sup>-methylnicotinamide,  
265

- Hodgson, Roland, Riker, A. J., and  
Peterson, W. H. Polysaccharide pro-  
duction by virulent and attenuated  
crown-gall bacteria, 89

- Hutchinson, M. C. See *Pillemer and  
Hutchinson*, 299

## I

- Irby, Virginia. See *Albanese*, 101

## J

- John, Hedda M. See *Nachmansohn  
and John*, 157  
Johnson, B. Connor, Hamilton, T. S.,  
and Mitchell, H. H. The excretion of  
pyridoxine, "pseudopyridoxine," and  
4-pyridoxic acid in the urine and sweat  
of normal individuals, 619

## K

- Kalckar, Herman M. Enzymatic micro-  
analysis of purine compounds, 313

- Enzymatic synthesis of a nucleoside,  
723

- Koch, F. C. See *White and Koch*, 535

- Kramer, Benjamin. See *Sobel, Rocken-  
macher, and Kramer*, 475

- Krehl, W. A., and Elvehjem, C. A. The  
importance of "folic acid" in rations  
low in nicotinic acid, 173

- Krejci, Laura E., Sweeny, Lucile, and  
Sanigar, Edward B. The electro-

- phoretic analysis of stored liquid hu-  
man plasmas, 693

- Kreke, Cornelius W., Bartlett, Sister  
Mary Domitilla, and Smalt, Sister  
Marie Augustine. An accelerator of  
catalase activity, 469

- Krueger, Keatha, and Peterson, W. H.  
The response of *Lactobacillus casei*  
and *Streptococcus faecalis* to vitamin  
B<sub>12</sub> and thymine, 145

## L

- Lamfrom, Hildegard. See *van Wagten-  
donk and Lamfrom*, 421

- Lavin, George I. See *Zamecnik, Lavin,  
and Bergmann*, 537

- Levin, Louis. The isolation of  $\alpha$ -  
estradiol from the urine of stallions,  
725

- Lewis, Howard B. See *Dzicwiatkowski  
and Lewis*, 77

- Lifson, Nathan, and Lorber, Victor.  
The time required for establishment of  
the thermal steady state in the Hill-  
Baltes apparatus, with application to  
the more rapid determination of os-  
motic activity, 209

- , —, and Hill, Edward L. Determina-  
tion of deuterium oxide by means of the  
Hill-Baltes vapor tension apparatus,  
219

- Lipmann, Fritz, and Tuttle, L. Con-  
stance. On the condensation of acetyl  
phosphate with formate or carbon  
dioxide in bacterial extracts, 505

- See *Utter, Lipmann, and Werkman*,  
521

- Lorber, Victor. See *Lifson and Lorber*,  
209

- See *Lifson, Lorber, and Hill*, 219

- Luck, James Murray. See *Rice, Ballou,  
Boyer, Luck, and Lum*, 609

- Luckey, T. D. See *Briggs, Luckey,  
Elvehjem, and Hart*, 303

- Luecke, R. W., and Pearson, P. B.  
Effect of the ingestion of excessive  
quantities of choline on the amount in  
tissues and urine, 561

- Lum, Funston G. See *Rice, Ballou,  
Boyer, Luck, and Lum*, 609

## M

- MacFadyen, Douglas A. Estimation of formaldehyde in biological mixtures, 107
- Marshall, Charles R. See *Marshall and Marshall*, 187
- Marshall, Wilfred, and Marshall, Charles R. The action of nitrites on blood, 187
- Mason, Harold L. Isolation of a urinary steroid with an oxygen atom at carbon 11, 719
- Medes, Grace. See *Weinhouse, Medes, and Floyd*, 411
- Melnick, Daniel. See *Hochberg, Melnick, and Oser*, 265
- Michaud, L. See *Ruegamer, Michaud, and Elvehjem*, 573
- Miltzer, Walter E. The inhibition of carbohydrate oxidations by borate, 247
- Miller, Russell C. See *Pence, Miller, Dutcher, and Thorp*, 647
- Milone, Henry S., Pesare, Pasquale J., and Veitch, Fletcher P., Jr. Enhancement of antibody production, 533
- See *Veitch and Milone*, 61
- Mitchell, H. H., Hamilton, T. S., Steggerda, F. R., and Bean, H. W. The chemical composition of the adult human body and its bearing on the biochemistry of growth, 625
- See *Johnson, Hamilton, and Mitchell*, 619
- Morales, Manuel F., Rathbun, Edith N., Smith, Robert E., and Pace, Nello. Studies on body composition. II. Theoretical considerations regarding the major body tissue components, with suggestions for application to man, 677
- Moses, Sylvia G. See *Herrmann and Moses*, 33, 47
- Muraschi, Thelma F. See *Quigley and Muraschi*, 463

## N

- Nachmansohn, David, and John, Hedda M. Studies on choline acetylase. I.

Effect of amino acids on the dialyzed enzyme. Inhibition by  $\alpha$ -keto acids, 157

- Nachmansohn, David, and Rothenberg, Mortimer A. Studies on cholinesterase. I. On the specificity of the enzyme in nerve tissue, 653
- Neuberger, A. The lysine content of casein and zein, 717
- Norris, Earl R., and Benoit, George J., Jr. Studies on trimethylamine oxide. I. Occurrence of trimethylamine oxide in marine organisms, 433
- and —. III. Trimethylamine oxide excretion by the rat, 443
- and Simmons, Richard W. The hematopoietic activity of xanthopterin in young salmon, 449
- See *Benoit and Norris*, 439
- Norris, L. C. See *Scott, Norris, Heuser, and Bruce*, 291

## O

- Oser, Bernard L. See *Hochberg, Melnick, and Oser*, 265

## P

- Pace, Nello, and Rathbun, Edith N. Studies on body composition. III. The body water and chemically combined nitrogen content in relation to fat content, 685
- See *Morales, Rathbun, Smith, and Pace*, 677
- See *Rathbun and Pace*, 667
- Page, Irvine H. See *Plentl and Page*, 49
- Parrish, D. B. See *Caldwell and Parrish*, 181
- Pearson, P. B. See *Luecke and Pearson*, 561
- Pence, James W., Miller, Russell C., Dutcher, R. Adams, and Thorp, William T. S. The thiamine content of pig blood, 647
- Pesare, Pasquale J. See *Milone, Pesare, and Veitch*, 533
- Peterson, W. H. See *Hodgson, Riker, and Peterson*, 89
- See *Krueger and Peterson*, 145

- Pillemer, Louis, and Hutchinson, M. C.  
The determination of the albumin and  
globulin contents of human serum by  
methanol precipitation, 299
- Plenti, Albert A., and Page, Irvine H.  
The purification of angiotonin, 49

## Q

- Quigley, James J., and Muraschi,  
Thelma F. Amino acid inhibition of  
copper proteinate formation, 463

## R

- Rathbun, Edith N., and Pace, Nello.  
Studies on body composition. I. The  
determination of total body fat by  
means of the body specific gravity, 667  
— See *Morales, Rathbun, Smith, and  
Pace*, 677  
— See *Pace and Rathbun*, 685
- Rice, Randall G., Ballou, Gerald A.,  
Boyer, Paul D., Luck, James Murray,  
and Lum, Funston G. The papain  
digestion of native, denatured, and  
"stabilized" human serum albumin,  
609
- Riker, A. J. See *Hodgson, Riker, and  
Peterson*, 89
- Rittenberg, D. See *Shemin and Ritten-  
berg*, 71
- Rockenmacher, Morris. See *Sobel,  
Rockenmacher, and Kramer*, 475
- Rothenberg, Mortimer A. See *Nach-  
mansohn and Rothenberg*, 653
- Rubin, Saul H., and De Ritter, Elmer.  
The adsorption of riboflavin on florisl,  
639
- Ruegamer, W. R., Michaud, L., and  
Elvehjem, C. A. A simplified method  
for the determination of iron in milk,  
573

## S

- Sanigar, Edward B. See *Krejci, Sweeny,  
and Sanigar*, 693
- Scott, M. L., Norris, L. C., Heuser, G.  
F., and Bruce, W. F. Studies on or-  
ganic factors required for prevention of  
anemia in chicks, 291

- Shemin, David, and Rittenberg, D. On  
the mechanism of the conversion of  
ornithine to proline *in vivo*; intramo-  
lecular nitrogen shift, 71
- Shive, William. See *Snell and Shive*,  
551
- Simmons, Richard W. See *Norris and  
Simmons*, 449
- Sims, E. A. H. Microdetermination of  
glycocyamine and arginine by means of  
a synthetic ion exchange resin for  
chromatographic separation, 239
- Smalt, Sister Marie Augustine. See  
*Kreke, Bartlett, and Smalt*, 469
- Smith, Robert E. See *Morales, Rathbun,  
Smith, and Pace*, 677
- Snell, Esmond E. The vitamin B<sub>6</sub>  
group. VII. Replacement of vitamin  
B<sub>6</sub> for some microorganisms by d(-)-  
alanine and an unidentified factor from  
casein, 497  
— and Shive, William. Growth inhibi-  
tion by analogues of pantothenic acid.  
Pantothenyl alcohol and related com-  
pounds, 551  
— See *Cunningham and Snell*, 491
- Sobel, Albert E., Rockenmacher, Morris,  
and Kramer, Benjamin. Carbonate  
content of bone in relation to the com-  
position of blood and diet, 475
- Steggerda, F. R. See *Mitchell, Hamil-  
ton, Steggerda, and Bean*, 625
- Stephenson, Mary L. See *Zamecnik,  
Stephenson, and Cope*, 135
- Stern, Kurt G. See *Beloff and Stern*, 19  
— See *Wallerstein and Stern*, 1
- Sweeny, Lucile. See *Krejci, Sweeny,  
and Sanigar*, 693

## T

- Tarr, Rose Ruth. See *Hamilton*,  
375, 397
- Taussky, Hertha H. See *Bonsnes and  
Taussky*, 581
- Thomas, James M. See *Brown, Bina,  
and Thomas*, 455
- Thorp, William T. S. See *Pence, Miller,  
Dutcher, and Thorp*, 647
- Tuttle, L. Constance. See *Lipmann and  
Tuttle*, 505

## U

- Udenfriend, Sidney. See *Brodie and Udenfriend*, 705
- Utter, M. F., Lipmann, Fritz, and Werkman, C. H. Reversibility of the phosphoroclastic split of pyruvate, 521
- Uzman, Lahut. See *Christensen, Uzman, and Hegsted*, 279

## V

- Veitch, Fletcher P., Jr., and Milone, Henry S. Studies on the sex hormones. I. The quantitative estimation of estrone in pure solution, 61
- . See *Milone, Pesare, and Veitch*, 533

## W

- Wagner, Dorothy L. See *Albanese*, 101
- van Wagtenonk, Willem J., and Lamfrom, Hildegard. A dietary factor essential for guinea pigs. VI. Changes in the distribution of acid-soluble phosphorus in the muscle during a deficiency of the antistiffness factor, 421

- Wallerstein, James S., and Stern, Kurt G. On bisulfite as an inhibitor of carboxylase and the mechanism of glycerol fermentation, 1

- Watkins, Helen D. See *MacFadyen*, 107

- Weinhouse, Sidney, Medes, Grace, and Floyd, Norman F. Fatty acid metabolism. III. Reactions of carboxyl-labeled acetic acid in liver and kidney, 411

- Werkman, C. H. See *Utter, Lipmann, and Werkman*, 521

- White, Wilfred, and Koch, F. C. Interference by cystine with the quantitative nitroprusside test for methionine, 535

## Z

- Zamecnik, Paul C., Lavin, George I., and Bergmann, Max. Microphotometric determination of the rate of enzymatic proteolysis, 537
- , Stephenson, Mary L., and Cope, Oliver. Peptidase activity of lymph and serum after burns, 135

# INDEX TO SUBJECTS

## A

- Acetic acid: Butyl-, tertiary, metabolism, glucuronic acid conjugation, *Dziewiatkowski and Lewis*, 77  
 Carboxyl-labeled, liver and kidney, reactions, *Weinhouse, Medes, and Floyd*, 411  
 Trimethyl-, metabolism, glucuronic acid conjugation, *Dziewiatkowski and Lewis*, 77  
 Acetylase: Choline, *Nachmansohn and John*, 157  
 —, dialyzed, amino acids and  $\alpha$ -keto acid effect, *Nachmansohn and John*, 157  
 Acetyl phosphate: Condensation, bacterial extracts, formate and carbon dioxide effect, *Lipmann and Tuttle*, 505  
 Alanine: d(-)-, vitamin B<sub>6</sub> replacement, microorganisms, *Snell*, 497  
 Albumin: Blood serum, denaturation, urea and guanidine with sodium caprylate, effect, *Boyer*, 715  
 —, —, determination, methanol precipitation method, *Pillemer and Hutchinson*, 299  
 —, —, papain, digestion, *Rice, Ballou, Boyer, Luck, and Lum*, 609  
 Alkaloid(s): Cinchona, determination, *Brodie and Udenfriend*, 705  
 Alloxan: Determination and properties, *Archibald*, 347  
 Hypoglycemia, effect, *Banerjee*, 547  
 Amine: Trimethyl-, oxide, *Norris and Benoit*, 433  
 —, —, *Benoit and Norris*, 439  
 —, —, *Norris and Benoit*, 443  
 —, —, excretion, rat, *Norris and Benoit*, 443  
 —, —, marine organisms, *Norris and Benoit*, 433  
 —, —, salmon, origin, *Benoit and Norris*, 439

- Aminoacetophenone: *p*-, diazotized, vitamin B<sub>6</sub> determination, use, *Brown, Bina, and Thomas*, 455  
 Amino acid(s): Choline acetylase, dialyzed,  $\alpha$ -keto acid effect, *Nachmansohn and John*, 157  
 Copper proteinate formation, effect, *Quigley and Muraschi*, 463  
 d-, utilization, *Albanese*, 101  
 Tyrocidine, *Christensen, Uzman, and Hegsted*, 279  
 Anemia: Chick, organic factors, relation, *Scott, Norris, Heuser, and Bruce*, 291  
 Angiotonin: Purification, *Plentl and Page*, 49  
 Anhydrase: Carbonic, sulfanilamide effect, *Davenport*, 567  
 —, thiophene-2-sulfonamide effect, *Davenport*, 567  
 Antibody: Production, *Milone, Pesare, and Veitch*, 533  
 Antistiffness factor: Deficiency, muscle acid-soluble phosphorus, distribution, effect, *van Wagendonk and Lamfrom*, 421  
 Arginine: Determination, micro-, chromatographic, *Sims*, 239

## B

- Bacillus: See also *Lactobacillus*, *Streptococcus*  
 Bacteria: Crown-gall, polysaccharide production, *Hodgson, Riker, and Peterson*, 89  
 Extracts, acetyl phosphate condensation, formate and carbon dioxide effect, *Lipmann and Tuttle*, 505  
 Bisulfite: Carboxylase, effect, *Wallerstein and Stern*, 1  
 Glycerol fermentation relation, *Wallerstein and Stern*, 1  
 Blood: Bone carbonate, relation, *Sobel, Rockenmacher, and Kramer*, 475



**Blood—continued:**

- Nitrite effect, *Marshall and Marshall*, 187
- Thiamine, *Pence, Miller, Dutcher, and Thorp*, 647
- Uric acid, determination, *Brown*, 601
- Blood plasma: Glutamine, *Hamilton*, 397
- carboxyl nitrogen determination, gasometric, *Hamilton*, 375
- Phospholipids, turnover, choline effect, *Friedlander, Chaikoff, and Entenman*, 231
- Storage effect, *Krejci, Sweeney, and Sanigar*, 693
- Blood serum: Albumin, denaturation, urea and guanidine with sodium caprylate, effect, *Boyer*, 715
- , determination, methanol precipitation method, *Pillemer and Hutchinson*, 299
- , papain digestion, *Rice, Ballou, Boyer, Luck, and Lum*, 609
- Globulin, determination, methanol precipitation method, *Pillemer and Hutchinson*, 299
- Peptidase, burns, *Zamecnik, Stephenson, and Cope*, 135
- Body: Chemical composition, growth relation, *Mitchell, Hamilton, Steggerda, and Bean*, 625
- Composition, *Rathbun and Pace*, 667
- Morales, Rathbun, Smith, and Pace*, 677
- Pace and Rathbun*, 685
- Bone: Carbonate, blood and diet relation, *Sobel, Rockenmacher, and Kramer*, 475
- Borate(s): Carbohydrate oxidation, effect, *Militzer*, 247
- Burn(s): Blood serum peptidase, *Zamecnik, Stephenson, and Cope*, 135
- Lymph peptidase, *Zamecnik, Stephenson, and Cope*, 135
- Butylacetic acid: Tertiary, metabolism, glucuronic acid conjugation, *Dziewiatkowski and Lewis*, 77

**C**

- Carbohydrate(s): Oxidation, borate effect, *Militzer*, 247

- Carbonate: Bone, blood and diet relation, *Sobel, Rockenmacher, and Kramer*, 475
- Carbon dioxide: Acetyl phosphate condensation with, bacterial extracts, *Lipmann and Tuttle*, 505
- Carbonic anhydrase: Sulfanilamide effect, *Davenport*, 567
- Thiophene-2-sulfonamide effect, *Davenport*, 567
- Carboxylase: Bisulfite effect, *Wallerstein and Stern*, 1
- Co-. See Cocarboxylase
- Spleen extracts, carp, effect, *Beloff and Stern*, 19
- Carp: Spleen extracts, carboxylase and cocarboxylase, effect, *Beloff and Stern*, 19
- Casein: Factor, vitamin B<sub>6</sub> replacement, microorganisms, *Snell*, 497
- Lysine, *Neuberger*, 717
- Catalase: Accelerator, *Kreke, Bartlett, and Smalt*, 469
- Chick: Anemia, organic factors, relation, *Scott, Norris, Heuser, and Bruce*, 291
- Nutrition, vitamins B<sub>10</sub> and B<sub>11</sub>, *Briggs, Luckey, Elvehjem, and Hart*, 303
- Chloride(s): Tendons, *Eichelberger and Brown*, 283
- Choline: Acetylase, *Nachmansohn and John*, 157
- , dialyzed, amino acids and  $\alpha$ -keto acid effect, *Nachmansohn and John*, 157
- Blood plasma phospholipids, turnover, effect, *Friedlander, Chaikoff, and Entenman*, 231
- Tissue choline, ingestion effect, *Luecke and Pearson*, 561
- Urine choline, ingestion effect, *Luecke and Pearson*, 561
- Cholinesterase: Nerve, specificity, *Nachmansohn and Rothenberg*, 653
- Chorion: Gonadotropin, denaturation by urea, temperature coefficient, *Bischhoff*, 29
- Cinchona: Alkaloids, determination, *Brodie and Udenfriend*, 705

- Coccarboxylase: Spleen extracts, carp,  
effect, *Beloff and Stern*, 19
- Collagen: Nitrogen, tendons, *Eichelberger and Brown*, 283
- Copper proteinate: Formation, amino  
acid effect, *Quigley and Muraschi*,  
463
- Creatinine: Determination, colorimetric,  
Jaffe reaction, *Bonsnes and Taussky*,  
581
- Crown-gall: Bacteria, polysaccharide  
production, *Hodgson, Riker, and  
Peterson*, 89
- Cystine: Methionine determination,  
effect, *White and Koch*, 535
- Utilization, *Albanese*, 101
- Cytochrome: Oxidase, eye lens, activity,  
*Herrmann and Moses*, 47

## D

- Deuterium oxide: Determination, Hill-  
Balde apparatus, *Lifson, Lorber, and  
Hill*, 219
- Diet: Bone carbonate, relation, *Sobel,  
Rockenmacher, and Kramer*, 475
- Guinea pig, essential factor, *van Wag-  
endonk and Lamfrom*, 421

## E

- Enzyme: Nucleoside synthesis, *Kalck-  
kar*, 723
- Phosphorylase *a* conversion to phos-  
phorylase *b*, *Cori and Cori*, 321
- Proteolysis rate, determination,  
micro-photometric, *Zamecnik,  
Lavin, and Bergmann*, 537
- Purine compounds, determination, mi-  
cro-, *Kalckkar*, 313
- Thymus nucleohistone degradation,  
*Cohen*, 255
- See also Acetylase, Anhydrase, Car-  
boxylase, Catalase, Cholinesterase,  
Coccarboxylase, Oxidase, Pápain,  
Peptidase, Phosphorylase
- Esterase: Cholin-. See Cholinesterase
- Estradiol:  $\alpha$ -, urine, isolation, *Levin*,  
725
- Estrone: Determination, *Veitch and  
Milone*, 61
- Eye: Glutathione, *Herrmann and Moses*,  
33

## Eye—continued:

- Lens, cytochrome oxidase activity,  
*Herrmann and Moses*, 47

## F

- Fat(s): Body, determination, body spe-  
cific gravity use, *Rathbun and Pace*,  
667
- , water and nitrogen relation, *Pace  
and Rathbun*, 685
- Tendons, *Eichelberger and Brown*, 283
- Fatty acid(s): Metabolism, *Weinhouse,  
Medes, and Floyd*, 411
- Fish: See also Carp, Salmon
- Florisil: Riboflavin adsorption, *Rubin  
and De Ritter*, 639
- Folic acid: Nicotinic acid-low rations,  
effect, *Krehl and Elvehjem*, 173
- Formaldehyde: Biological mixtures, de-  
termination, *MacFadyen*, 107
- Formate: Acetyl phosphate condensation  
with, bacterial extracts, *Lipmann  
and Tuttle*, 505

## G

- Globin: Myo-, synthetic, crystalline,  
*Drabkin*, 721
- Unaltered, *Drabkin*, 721
- Globulin: Blood serum, determination,  
methanol precipitation method, *Pil-  
lemer and Hutchinson*, 299
- Glucuronic acid: Pivalic acid metabo-  
lism, conjugation, *Dziewiatkowski  
and Lewis*, 77
- Trimethylacetic and butylacetic acid,  
tertiary, metabolism, conjugation,  
*Dziewiatkowski and Lewis*, 77
- Glutamine: Blood plasma, *Hamilton*,  
397
- Carboxyl nitrogen, blood plasma and  
tissue, determination, gasometric,  
*Hamilton*, 375
- Tissues, *Hamilton*, 397
- Glutathione: Eye, *Herrmann and Moses*,  
33
- Glycerol: Fermentation mechanism, bi-  
sulfite and carboxylase relation, *Wal-  
lerstein and Stern*, 1
- Glycocyamine: Determination, micro-,  
chromatographic, *Sims*, 239

- Gonadotropin:** Chorionic, denaturation by urea, temperature coefficient, *Bischoff*, 29
- Gramicidin:** Hydrolysis, acid, *Christensen and Hegsted*, 593
- Growth:** Body composition, relation, *Mitchell, Hamilton, Steggerda, and Bean*, 625
- Pantothenic acid analogues, effect, *Snell and Shive*, 551
- Pantotheryl alcohol and related compounds, effect, *Snell and Shive*, 551
- Guanidine:** Blood serum albumin denaturation, sodium caprylate effect, *Boyer*, 715
- Guinea pig:** Diet essential, *van Wagten-donk and Lamfrom*, 421

## H

- Heart:** Muscle, tyramine effect, *in vitro*, *Bernheim and Bernheim*, 425
- Hematopoiesis:** Salmon, xanthopterin effect, *Norris and Simmons*, 449
- Hill-Baldes:** Apparatus, deuterium oxide determination, *Lifson, Lorber, and Hill*, 219
- , steady state time, osmotic activity determination by, *Lifson and Lorber*, 209
- Histone:** Nucleo-. See Nucleohistone
- Hormone(s):** Sex. See Sex, hormones
- Hypoglycemia:** Alloxan effect, *Banerjee*, 547

## I

- Ion(s):** Exchange, resin, glycocyamine and arginine determination, micro-, chromatographic, *Sims*, 239
- Iron:** Milk, determination, *Ruegamer, Michaud, and Elvehjem*, 573

## K

- Keto acid(s):**  $\alpha$ -, choline acetylase, dialyzed, effect, *Nachmansohn and John*, 157
- Kidney:** Acetic acid, carboxyl-labeled, reactions, *Weinhouse, Medes, and Floyd*, 411

## L

- Lactobacillus casei:** Thymine effect, *Krueger and Peterson*, 145
- Vitamin B<sub>6</sub> effect, *Krueger and Peterson*, 145
- Lens:** Eye, cytochrome oxidase activity, *Herrmann and Moses*, 47
- Light:** Vitamin A determination, Carr-Price reagent, effect, *Caldwell and Parrish*, 181
- Lipid(s):** Phospho-. See Phospholipid
- Liver:** Acetic acid, carboxyl-labeled, reactions, *Weinhouse, Medes, and Floyd*, 411
- Phenolic compounds, metabolism *in vitro*, scurvy, *Darby, DeMeio, Bernheim, and Bernheim*, 67
- Lymph:** Peptidase, burns, *Zamecnik, Stephenson, and Cope*, 135
- Lysine:** Casein, *Neuberger*, 717
- Zein, *Neuberger*, 717

## M

- Marine organism(s):** Trimethylamine oxide, *Norris and Benoit*, 433
- Methionine:** Determination, cystine effect, *White and Koch*, 535
- Methylnicotinamide:** N<sup>1</sup>-, urine, determination, chemical, *Hochberg, Melnick, and Oser*, 265
- Microorganism(s):** Vitamin B<sub>6</sub> replacement, d(-)-alanine and casein factor, *Snell*, 497
- Milk:** Iron, determination, *Ruegamer, Michaud, and Elvehjem*, 573
- Muscle:** Phosphorus, acid-soluble, distribution, antistiffness factor deficiency, effect, *van Wagten-donk and Lamfrom*, 421
- Phosphorylase  $\alpha$ , stimulation and recovery, effect, *Cori*, 333
- See also Heart
- Myoglobin:** Synthetic, crystalline, *Drabkin*, 721

## N

- Nerve:** Cholinesterase specificity, *Nachmansohn and Rothenberg*, 653
- Nicotinamide:** N<sup>1</sup>-Methyl-, urine, determination, chemical, *Hochberg, Melnick, and Oser*, 265

- Nicotinic acid: -Low rations, folic acid effect, *Krehl and Elvehjem*, 173
- Nitrite(s): Blood composition, effect, *Marshall and Marshall*, 187
- Nitrogen: Body, body fat, relation, *Pace and Rathbun*, 685
- Carboxyl, glutamine, blood plasma and tissue, determination, gasometric, *Hamilton*, 375
- Collagen, tendons, *Eichelberger and Brown*, 283
- Intramolecular shift, *Shemin and Rittenberg*, 71
- Total, tendons, *Eichelberger and Brown*, 283
- Nucleohistone: Thymus, degradation, enzymatic, *Cohen*, 255
- Nucleoside(s): Synthesis, enzymatic, *Kalckar*, 723
- Nutrition: Chick, vitamins B<sub>12</sub> and B<sub>11</sub>, *Briggs, Luckey, Elvehjem, and Hart*, 303

## O

- Organic compound(s): Basic, determination, *Brodie and Udenfriend*, 705
- Organic factor(s): Anemia, chick, relation, *Scott, Norris, Heuser, and Bruce*, 291
- Ornithine: Proline, conversion mechanism in vivo, *Shemin and Rittenberg*, 71
- Osmotic activity: Determination, Hill-Baldes apparatus, *Lifson and Lorber*, 209
- Oxidase: Cytochrome, eye lens, activity, *Herrmann and Moses*, 47

## P

- Pantothenic acid: Analogues, growth, effect, *Snell and Shive*, 551
- Pantothenyl alcohol: Growth effect, *Snell and Shive*, 551
- Related compounds, growth effect, *Snell and Shive*, 551
- Papain: Blood serum albumin, digestion by, *Rice, Ballou, Boyer, Luck, and Lum*, 609
- Peptidase: Blood serum, burns, *Zamecnik, Stephenson, and Cope*, 135

## Peptidase—continued:

- Lymph, burns, *Zamecnik, Stephenson, and Cope*, 135
- Perspiration: Pyridoxine, pseudopyridoxine, and 4-pyridoxic acid excretion, *Johnson, Hamilton, and Mitchell*, 619
- Phenolic compound(s): Metabolism, liver, in vitro, scurvy, *Darby, DeMeio, Bernheim, and Bernheim*, 67
- Phospholipid(s): Blood plasma, turnover, choline effect, *Friedlander, Chailoff, and Entenman*, 231
- Phosphorus: Acid-soluble, muscle, distribution, antistiffness factor deficiency, effect, *van Wageningen and Lamfrom*, 421
- Phosphorylase(s): a and b, diffusion constant and electrophoretic mobility, *Green*, 315
- , conversion to phosphorylase b, enzymatic, *Cori and Cori*, 321
- , muscle, stimulation and recovery, effect, *Cori*, 333
- b, activity and crystallization, *Cori and Cori*, 341
- Pivalic acid: Metabolism, glucuronic acid conjugation, *Dziewiatkowski and Lewis*, 77
- Polysaccharide(s): Crown-gall bacteria, production, *Hodgson, Riker, and Peterson*, 89
- Prolan: Inactivation, reversible, *Bischhoff*, 577
- Proline: Ornithine conversion mechanism in vivo, *Shemin and Rittenberg*, 71
- Protein(s): Mixtures, electrophoresis, *Cooper*, 727
- Proteolysis: Enzyme, rate, determination, microphotometric, *Zamecnik, Lavin, and Bergmann*, 537
- Pseudopyridoxine: Sweat, excretion, *Johnson, Hamilton, and Mitchell*, 619
- Urine, excretion, *Johnson, Hamilton, and Mitchell*, 619
- Purine compound(s): Determination, micro-, enzymatic, *Kalckar*, 313
- Pyridoxal: Stability, *Cunningham and Snell*, 491



—continued:

- Methylnicotinamide, determination, chemical, *Hochberg, Melnick, and Oser*, 265  
 ridoxine, pseudopyridoxine, and 4-pyridoxic acid excretion, *Johnson, Hamilton, and Mitchell*, 619  
 roid, isolation, *Mason*, 719

## V

- ain(s): A, determination, Carr-Price reagent, light effect, *Caldwell and Parrish*, 181  
*Lactobacillus casei*, effect, *Krueger and Peterson*, 145  
*Streptococcus faecalis*, effect, *Krueger and Peterson*, 145  
 . See also Thiamine  
 . See also Riboflavin  
*Cunningham and Snell*, 491  
*Snell*, 497  
 , determination, *p*-aminoacetophenone, diazotized, use, *Brown, Bina, and Thomas*, 455

Vitamin(s)—continued:

- B<sub>6</sub>, replacement, *d*(-)-alanine and casein factor, microorganisms, *Snell*, 497  
 —. See also Pyridoxal, Pyridoxamine, Pyridoxine  
 B<sub>10</sub>, chick nutrition, *Briggs, Luckey, Elvehjem, and Hart*, 303  
 B<sub>11</sub>, chick nutrition, *Briggs, Luckey, Elvehjem, and Hart*, 303

## W

- Water: Body, body fat, relation, *Pace and Rathbun*, 685  
 Tendons, *Eichelberger and Brown*, 283

## X

- Xanthopterin: Hematopoiesis, salmon, effect, *Norris and Simmons*, 449

## Z

- Zein: Lysine, *Neuberger*, 717